UAB
Universitat Autònoma de Barcelona

Toxicological assessment of nanoplastics using immune cells: a study on polystyrene and polyethylene terephthalate nanoplastics

Alireza Tavakolpournegari

Toxicological assessment of nanoplastics using immune cells: a study on polystyrene and polyethylene terephthalate nanoplastics

Alireza Tavakolpournegari

Universitat Autònoma de Barcelona

Facultat de Biociències

Departament de Genètica i de Microbiologia

Grup de Mutagènesi

Toxicological assessment of nanoplastics using immune cells: a study on polystyrene and polyethylene terephthalate nanoplastics

DOCTORAL DISSERTATION

Alireza Tavakolpournegari

2024

Universitat Autònoma de Barcelona

Facultat de Biociències

Departament de Genètica i de Microbiologia

Grup de Mutagènesi

Toxicological assessment of nanoplastics using immune cells: a study on polystyrene and polyethylene terephthalate nanoplastics

Dissertation respectfully submitted by

Alireza Tavakolpournegari

To Universitat Autònoma de Barcelona in partial fulfillment of the requirements for the degree of Doctor of Philosophy, as per the Doctorate Program in Genetics

Under the direction of Dr. Alba Hernández Bonilla and Dr. Balasubramanyam Annangi

Dr. Alba Hernández Bonilla **Dr. Balasubramanyam Annangi**

Acknowledgments

The PhD journey is far from a straightforward path in academic progress, often bringing with it challenges, anxieties, and pressures. Navigating this path would not have been possible without the support of everyone who accompanied me throughout this period. Therefore, I want to extend my sincere gratitude to all those who have played a role in my PhD journey and PhD life.

First and foremost, I am profoundly grateful to Dr. Alba Hernandez, who has been much more than a supervisor. Alongside fostering my scientific and professional growth, she consistently encouraged and supported my aspirations in this field, always keeping her time flexible for guidance despite her countless responsibilities. I also feel deeply honored to have had the opportunity to work with Prof. Ricard Marcos. His critical insights and disciplined approach to research helped me develop a foundation of independent thinking. Additionally, I extend heartfelt thanks to Dr. Subramanyam Annangi for his invaluable and friendly advice on my projects, which greatly contributed to my development as a professional researcher.

My gratitude also goes to Susana Pastor, whose assistance was invaluable during my initial project stages. I learned so much from her during that time. Furthermore, I thank Dr. Qiou Wei, my supervisor during my international stay at the University of Kentucky, whose dedication to toxicology and cancer biology helped me gain essential skills and structured insights in the field.

I am also grateful to Gloria Umbert and Cristian Valliente for their infectious enthusiasm and helpful advice during my time in the lab of mutagenesis. I

am incredibly appreciative of the ongoing support from all staff members in the Department of Genetics and Microbiology throughout my PhD, and to the Generalitat de Catalunya for providing essential financial support that allowed me to focus fully on my thesis. This work was also made possible through the guidance of Dr. Alba Hernandez and financial assistance from PlasticHeal (Innovative tools to study the impact and mode of action of micro and nanoplastics on human health: towards a knowledge base for risk assessment H2020-965196), the Generalitat de Catalunya, and the Spanish Ministry of Science and Innovation (project 2021-SGR-00731 and PID2020-116789, RB-C43).

To my lab mates, I owe a debt of gratitude for their teamwork and creative ideas, which elevated the quality of my research and created a supportive, collaborative environment. Special thanks to Aliro Villacorta, who has become more than a colleague—his advice, both inside and outside the lab, was always invaluable. I am thankful for the opportunity to work and share PhD life with Michelle Morataya, Javi Gutierrez, Gooya Banaei, and Juan Martin, whose camaraderie I will miss, along with our coffee breaks and popcorn after work.

I am also grateful to the postdoc fellows in the mutagenesis lab, particularly Dr. Alba Garcia, who provided generous support throughout various stages of my PhD journey. I will never forget her kindness and guidance. I am equally thankful to have met Mohamed Alaraby, Laura Rubio, and Jessica Arribas, whose knowledge and experience were a source of inspiration for me.

My progress and success in this PhD, as well as my drive to continue in this field, are largely due to the unwavering support of my beloved family and

friends. Despite the distance, their encouragement has been a constant presence in my life. To my father, Masoudali Tavakolpournegari, my mother, Akram Dorri, and my sister, Ayda Tavakolpournegari, who sent the warmth of family across the miles—I owe all that I have achieved to them. I am also deeply grateful to my friends Arash Matinahmadi and Seyed Hesamoddin Bidooki, whose steadfast friendship since 2012 has been a pillar of strength. My uncle, Harry Dorri, also deserves thanks for his invaluable advice that helped me find my way far from home.

Finally, to anyone who has taken the time to read this far, please know that everyone who has been part of this journey has my deepest appreciation. Each of you has contributed to shaping the person who is writing these words today.

Thank you.

ABSTRACT

The environmental presence of micro/nanoplastics (MNPLs) is a growing concern due to their potential implications for human health and ecosystems. MNPLs can originate from two primary sources: the physicochemical and biological degradation of larger plastic items (secondary MNPLs) or through industrial processes that manufacture these materials at the nanoscale for various commercial applications (primary MNPLs). The toxicological profile of MNPLs is influenced by their size and the capacity of cells and organisms to internalize them, raising questions about their health risks and biological effects. This dissertation aims to explore the multifaceted aspects of nanotoxicology concerning MNPLs, emphasizing their interactions with biological systems and potential health hazards.

Two studies are presented to elucidate these interactions. The first study examines the biological effects of polystyrene MNPLs of varying sizes (50, 200, and 500 nm) on three different human hematopoietic cell lines: Raji-B, THP-1, and TK6. While none of the MNPL sizes induced toxicity or affected cell growth, significant internalization was observed, particularly in Raji-B and THP-1 cells, with uptake inversely related to particle size. Notably, mitochondrial membrane potential loss demonstrated dose-related effects in Raji-B and THP-1 cells, and cell type/particle size dependent ROS were recorded in response to PSNPLs exposure. This study highlights the importance of size, biological endpoints, and cell type in modulating the toxicological profile of MNPLs.

The second study focuses on the health risks posed by secondary MNPLs, particularly polyethylene terephthalate (PET) NPLs resulting from the degradation of plastic water bottles. Utilizing mouse alveolar macrophages (MH-S) as the target cells, this investigation concentrated on the cells that internalized the PETNPLs, providing a realistic assessment of their potential adverse effects. The study employed various biomarkers, including intracellular reactive oxygen species (ROS) levels, mitochondrial membrane potential variations, and macrophage polarization to M1 (pro-inflammatory) and M2 (anti-inflammatory) states. Results indicated that, despite complete internalization at high

concentrations (up to 100 μg/mL), PETNPLs did not induce toxicity. However, increased ROS levels and alterations in mitochondrial potential were observed, particularly after 24 hours of exposure. Furthermore, macrophage polarization to M1 was pronounced at both exposure times, while M2 polarization was only evident after prolonged exposure.

Together, these studies underscore the need for a deeper understanding of the health implications associated with MNPL exposure, particularly as nanotoxicology continues to evolve in response to the pervasive presence of these materials in our environment. The findings emphasize that size, exposure duration, and the specific biological context play critical roles in determining the toxicological effects of MNPLs, paving the way for future research into the mechanisms underlying their interactions with biological systems.

ABBREVIATION LIST

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
DCFH-DA	2’,7’-dichlorodihydrofluorescein diacetate
DHE	Dihydroethidium
DLS	Dynamic light scattering
ENMs	Engineered nanomaterials
ENPs	Engineered nanoparticles
ER	Endoplasmic reticulum
FTIR	Fourier-transform infrared spectroscopy
iDye-PETNPLs	iDye labeled polyethylene terephthalate nanoplastics
iDye-PSNPLs	iDye labeled polystyrene nanoplastics
iROS	Intracellular reactive oxygen species
MH-S	Alveolar Macrophage Cells
MMP	Mitochondrial membrane potential
MNPLs	Micro- and nanoplastics
MPLs	Microplastics
NPLs	Nanoplastics
NPs	Nanoparticles
PBS	Phosphate-buffered saline
PET	Polyethylene terephthalate
PETNPLs	Polyethylene terephthalate nanoplastics
PLA	Polylactic acid
PP	Polypropylene
PS	Polystyrene
PSNPLs	Polystyrene nanoplastics
PVC	Polyvinylchloride

Raji-B	B lymphocyte ells
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
THP-1	Human leukemia monocytic cells
TK6	Lymphoblast cells

INDEX

1. Introduction 1

1.1. Plastics, history and application 2

1.2. Plastics as a global concern 4

1.2.1. Plastics among other polymers 5

1.2.2. Plastics in the environment 5

1.2.3. Micro- and nanoplastics, sources and occurrence 6

1.3. Human exposure to micro- and nanoplastics 13

1.3.1. Ingestion 14

1.3.2. Inhalation 16

1.3.3. Dermal contact 16

1.3.4. Nanoplastic translocation through human barriers 17

1.3.5. Factors affecting nanoplastic translocation 18

1.4. Potential impacts of MNPLs on human health 19

1.4.1. Polystyrene nanoplastics (PSNPLs) 20

1.4.2. Polyethylene terephthalate nanoplastics (PETNPLs) 21

1.4.3. Different types of nanoplastics for biological studies 22

1.4.4. Impact of MNPLs on blood system 24

1.5. Nanoplastics and Immunotoxicity 25

1.5.1. Overview of immune system mechanism 25

1.5.2. Immune response toward MNPLs exposure 25

1.5.3. *In vivo* studies 26

1.5.4. *In vitro* studies 28

1.5.5. The THP-1 cell line 28

1.5.6. The TK6 cell line 29

1.5.7. The Raji-B cell line 30

1.5.8. The MH-S cell line 31
1.6. Research gaps in studying the immunotoxicity of MNPLs 32
1.6.1. Size effects 33
1.6.2. Type of plastic polymer 33
1.6.3. Cell type and immunotoxic endpoints 34
2. Objectives 36
3. Results 39
3.1. Chapter 1 (Article1) 40
3.2. Cahpter2 (Article2) 40
4. Discussion 42
4.1. Hazardous effects of PSNPLs with different sizes on human immune cell lines 45
4.1.1. Particle size and cell type affect the interaction between PSNPLs and hematopoietic cell lines 47
4.1.2. Toxic response to PSNPLs is associated with the particle size and cell type influence 49
4.2. Harmful effects of true-to-life nanoplastics derived from PET water bottles in alveolar macrophages 52
4.2.1. The importance of using true-to-life nanopastics 52
4.2.2. PETNPLs affect immune system through inhalation 54
4.2.3. PETNPLs exposure leads to macrophage polarization in MH-S cells 57
5. Conclusions 61
6. References 64
7. Annexes 86

7.1. The pdf of the published paper entitled Hazard assessment of different-sized polystyrene nanoplastics in hematopoietic human cell lines 88
7.1.1. Supplementary figures related to the published paper entitled Hazard assessment of different-sized polystyrene nanoplastics in hematopoietic human cell lines 99
7.2. The pdf of the published paper entitled Harmful effects of true-to-life nanoplastics derived from PET water bottles in alveolar macrophages 103
7.2.1. The pdf of the corrigendum to “Harmful effects of true-to-life nanoplastics derived fromPET water bottles in human alveolar macrophages” 116
7.2.2. Supplementary figures related to the published paper entitled Harmful effects of true-to-life nanoplastics derived from PET water bottles in alveolar macrophages 118

1. Introduction

1. INTRODUCTION

1.1. Plastics, history and applications

Plastic refers to a synthetic material manufactured by repeating organic monomers (resins, elastomers, artificial fibers, among others). The historical background of today's known plastic dates back to 1907 when Leo Hendrick Baekeland used the name plastic for the first time after he invented Bakelite, the first known synthetic plastic (Williams & Rangel-Buitrago, 2022). The journey of plastic was initiated in the mid-nineteenth century in London by Alexander Parkes after introducing Parkesine, a celluloid-based material that started the domino in plastic production and innovation. In the early 20th century, other types of plastics were produced including polystyrene (PS) in 1920 which made a revolution in food packaging, then the most famous and applicable plastic polymer called polyethylene (PE) was produced in 1933 and used in various products (*Handbook of Polymer Applications in Medicine and Medical Devices*, 2014). In 1933, the B.F. Goodrich Company moved one step ahead by developing plasticization in this field and producing polyvinylchloride (PVC).

The application of plastic in all aspects of life turned this material into a versatile consuming product among people during the 20th to 21st century. Various physiochemical features of plastic, such as high electrical insulation, corrosion resistance, durability, lightweight and strong properties, and, most importantly, the low price of this material, have raised demands for this product globally. Packaging, transportation, clothing, telecommunication, medical, and technological applications have dramatically increased the production of plastic from 0.5 million tons in 1950 to 400 million tons in 2022 and Europe consists of over 58 million tons of this production meaning that this continent produced around 14% of the world's plastic (Andrady & Neal, 2009; Plasticseurope, 2023). In addition, China has produced the most plastic in the world accounting for 32% which along with Japan and other Asian countries consists of more than 54% percent of produced plastics in the globe (Figure 1). Among this production, 2.4 million tons of plastics were bio-based and bio-attributed plastics, and more than 35% of them have been made as post-consumer recycled plastics (PlasticsEurope, 2023).

INTRODUCTION

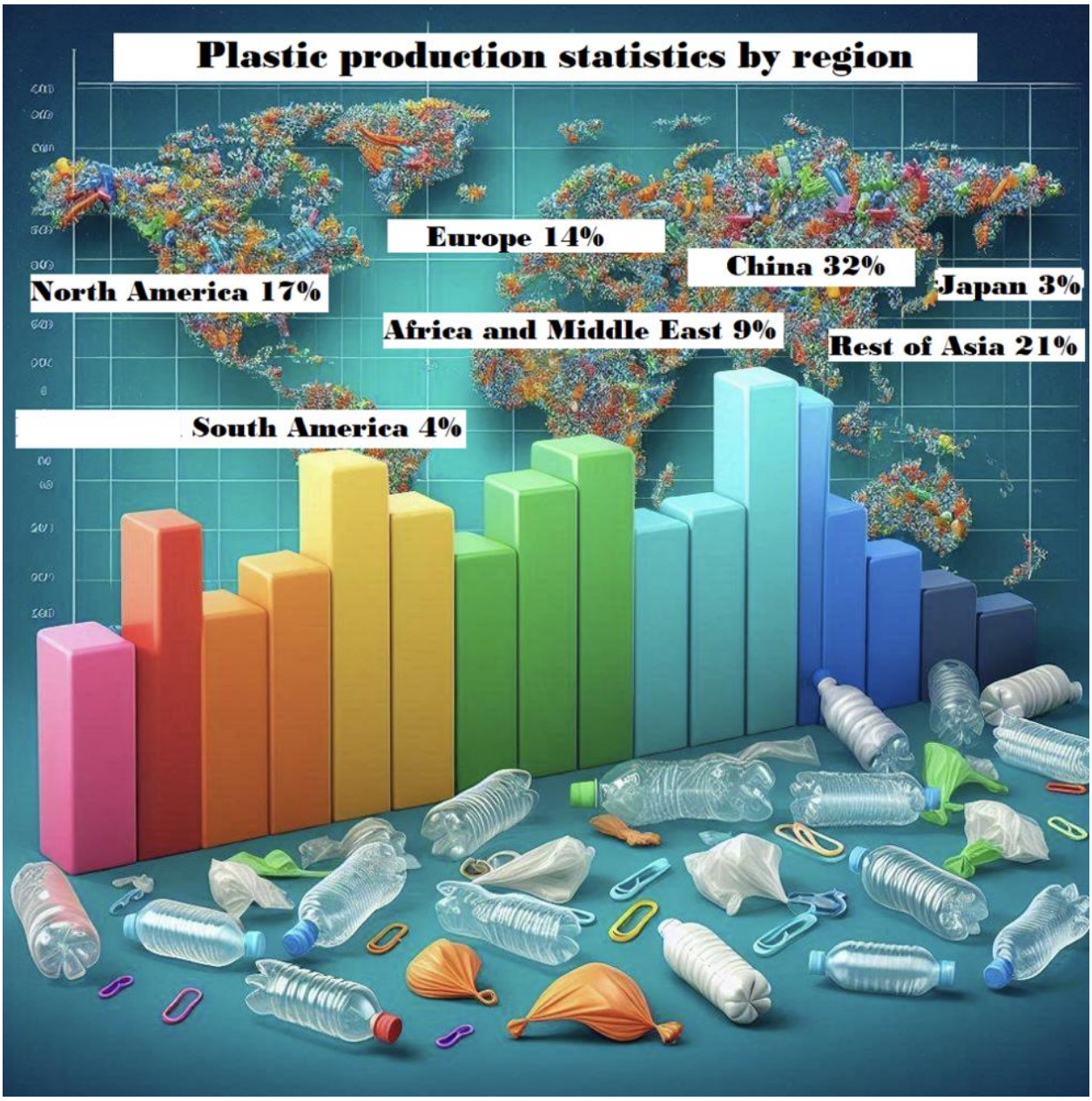


Figure 1. The statistics of plastic production worldwide. Data are separated by countries and continents (PlasticsEurope, 2023).

The area of plastic application shows a growing tendency by which we are observant of using plastic in various parts of our daily lives; among all these demands, most plastics are used in packaging covering 46% of the global distribution of plastics in various sectors (Figure 2) (Acharjee et al., 2023).

1.2. Plastics as a global concern

Plastic pollution has emerged as a critical global concern, with over 460 million metric tons of plastic produced annually. The ecological impact from such enormous plastic waste is extremely serious: it destroys the ecosystems, threatens wildlife, and contributes to climate change. This growing crisis underlines the need for the development and implementation of effective plastic waste management systems alongside drastic reduction in the generation of plastic waste. (Gautam Bhanu Pratap Singhand Qureshi, 2024; Karali et al., n.d.).

The environmental impact is profound, affecting marine life, terrestrial ecosystems, and human health. The urgency to address this issue has led to significant international efforts, including the United Nations Environment Assembly's resolution to develop a legally binding instrument to end plastic pollution by the end of 2024 ("On the Plastics Crisis," 2023). Recent initiatives highlight the global commitment to tackling plastic pollution. For instance, the fourth session of the Intergovernmental Negotiating Committee, held in Ottawa in April 2024, aimed to finalize a global treaty on plastic pollution. If agreed upon, this treaty would be a landmark decision, uniting countries worldwide in a shared goal to mitigate plastic pollution's adverse effects (*Pivotal Fourth Session of Negotiations on a Global Plastics*, 2024).These efforts reflect a growing recognition of the plastic crisis and the collective resolve to create a sustainable future.

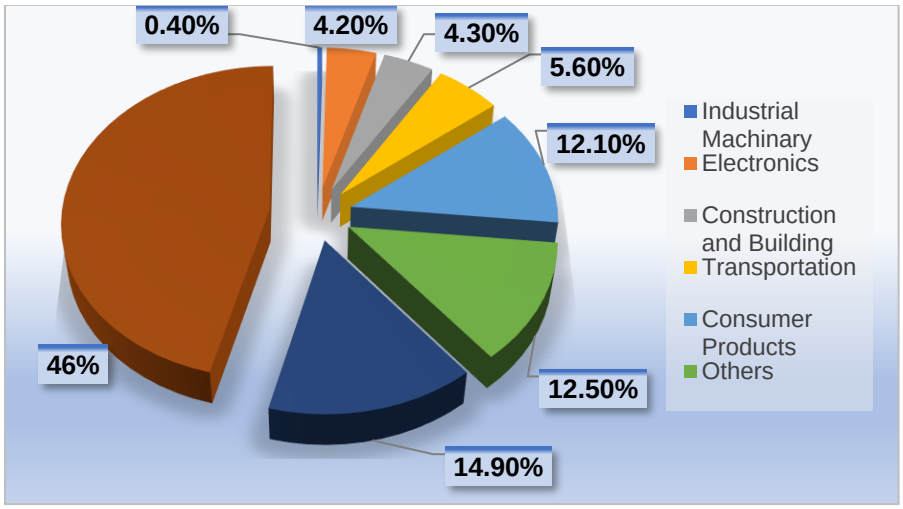


Figure 2. The distribution of plastic applications in different sectors.

1.2.1. Plastics among other polymers

Plastics can be defined as "*a wide range of synthetic or semi-synthetic materials that use polymers as a main ingredient. Their plasticity makes it possible for plastics to be molded, extruded or pressed into solid objects of various shapes".*

Different classifications for polymers rely on the monomers' nature and chemical properties, including origin, thermal, and mechanical properties, chain structure, molecular configuration, and end-use. Plastics are a specific kind of polymer that consists of organic monomers in their chemical properties, has a linear molecular structure, and shows two distinct thermal properties including thermoplastics and thermosets. The former is a type of plastic that undergoes melting upon heating and will be solid when cooled. On the other hand, thermosets are plastics that undergo forming and hardening by heating while they are infusible and do not reshape after heating (Desidery & Lanotte, 2022). Polystyrene (PS), polyethylene terephthalate (PET), polyvinyl chloride (PVC), and polypropylene (PP) are examples of thermoplastics, and polyurethane (PU), and phenol-formaldehyde (PF) are thermosets (Andrady, 2017).

1.2.2. Plastics in the environment

Despite the revolutionary role of plastics in human life, the lack of improper disposal of these materials in an environment caused a serious global concern due to their ability to accumulate in an environment which resulted from several reasons including, the high rate of production and demands of plastic, short life of the product usage and improper waste management and handling regarding plastics (Di et al., 2021; Napper et al., 2021; K. Zhang et al., 2021). In 1972, the first sparkles of research about plastic pollution and environmental accumulation were initiated by studying buoyant plastics in an ocean (Carpenter & Wolverton, 2017; Schwarz et al., 2019). estimated that a range of 250 metric kilotons of plastic is floated on the ocean's surface (Eriksen et al., 2014;Van Sebille et al., 2015); this estimation is much larger when considering that 800-2,400 kilotons of plastic

from rivers and 4,800-23,000 from coastal regions enter to the ocean annually Borrelle et al., 2020;Van Emmerik et al., 2023. Several studies focused on plastic pollution in the oceans by modeling their potential accumulation. Among them, Kaandorp et al. (2023) estimated the amount of plastic entering the ocean and the fate of the plastics in different aspects of the environment by a 3D map of the global ocean indicating the total global plastic mass in the sea by considering different routes of entry, plastic features and size, and other physiochemical reactions that occur for plastic wastes.

Most of the attention has gone to marine environments, although plastic pollution in terrestrial ecosystems also entails serious risks. Terrestrial environments are substantial sources and reservoirs of plastic litter, most of which migrates into rivers and oceans (Nizzetto et al., 2016). Research shows that about one-third of global plastic waste ends up in soil and freshwater systems**,** and terrestrial plastic pollution is often much higher than in marine environments, by four to 23 times**,** depending on the ecosystem. This highlights how plastic particles accumulate on land, especially in soils and sediments, where they can have toxic effects on organisms and disrupt ecological functions (Rai et al., 2023). specially, microplastics enter agricultural lands via irrigation, sewage sludge, and atmospheric deposition, disrupting the structure of soils and microbial communities therein. The globally estimated burden of plastic in terrestrial ecosystems is even higher than that in the oceans, considering that about 32% of all plastic waste generated annually remains on land (Rillig & Lehmann, 2020;Geyer et al., 2024)**.**

1.2.3. Micro- and nanoplastics, sources and occurrence

Primary MNPLs are those particles that are intentionally made small and then added directly into various consumer products. Classic examples include microbeads in cosmetic and personal care products, which are manufactured to perform exfoliation and cleaning. Many of these are then washed down the drains, contributing a great deal to the pollution of the environment. Other primary MNPLs come from industrial processes such as plastic beads used in various blasting activities and might be inadvertently released to the environment during their use.

On the contrary, secondary MNPLs are created through the breaking of larger plastic items called macroplastics. These are performed through several physical and chemical processes such as abrasion, photodegradation, and oxidative breakdown. During the breaking procedure, the macroplastics will release smaller particles into the environment, thereby contaminating it. Examples of secondary MNPLs would be microfibers shed from synthetic textiles during washing and fragmented plastic products such as bottles and packaging materials. These secondary particles are of particular concern since they can occur in very diverse environments: soils, freshwater, and marine ecosystems point to the pervasive nature of plastic pollution (Xayachak et al., 2024).

Most of the produced plastics are single-use, so they enter the environment after utilization because only 12% of plastic wastes undergo the recycling process and 9% are incinerated; Therefore, 79% of the remaining plastic is accumulated in the natural system (Lau et al., 2020; Geyer et al., 2017). However, it should be underlined that micro- and nanoplastics do not necessarily come only from improper waste disposal but could also result from the fragmentation of all types of plastics in all stages of their lifecycle. It can take place during activities related to material extraction, manufacturing, and transport and may further occur during use and at the end of life, including recycling and waste management. Plastic products are exposed, for example, even before they reach the consumer, to various mechanical stresses and environmental conditions that can result in microplastic shedding (Xayachak et al., 2024).Based on estimations, 19-23 million tons of plastic waste entered the aquatic system in 2016, and reached up to 53 million tons by 2023 (Borrelle et al., 2020). Once they are in the environment, they degrade to smaller fragments from macro size called macroplastic (>25 mm) to mesoplastics (5 mm-25 mm), and smaller particles known as microplastics (<5 mm) and nanoplastics (<1 µm) (Figure 3) (Hartmann et al., 2019). The presence of microplastics in nearly all types of environments has been demonstrated in studies, from marine ecosystems to freshwater and terrestrial habitats. As (Bellasi et al., 2020) have pointed out, microplastics are distributed throughout freshwater systems-from lakes and rivers into flowing sediments. The accumulation of plastics, therefore, presents microplastics in both marine and terrestrial ecosystems as a crucial contaminant. The MNPL's dispersion and

accumulation in the environment align with plastic production and consumption and convincing evidence indicates that due to their small size, they can accumulate in different corners of the Earth's environment including water, soil, rivers, seas, oceans, sediments, and even the atmosphere and biosphere (Pradel et al., 2023). Recent studies discovered the presence of micro- and nanoplastics in outdoor and indoor air environments and urban areas. (Torres-Agullo et al., 2022, Wright et al., 2020, Allen et al., 2019). For instance, Dris et al. (2016) reported the presence of plastic fibers in the atmosphere that originated from synthetic textiles.

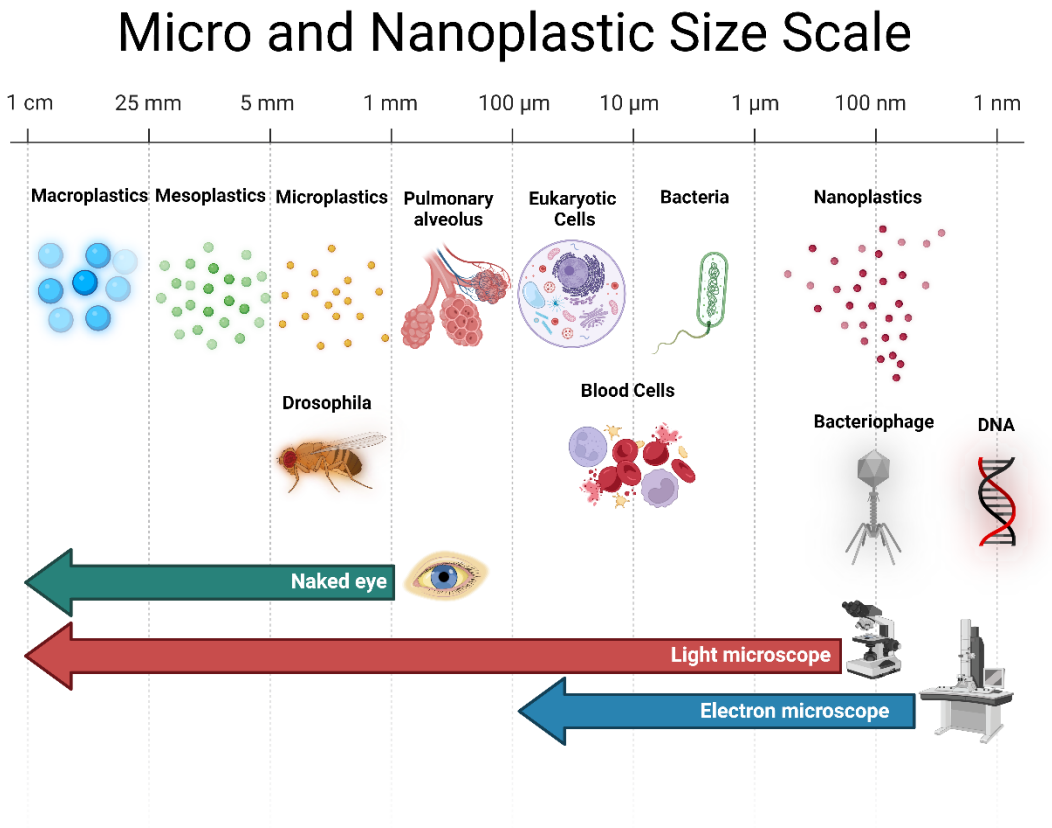


Figure 3. Schematic overview of the Micro and Nanoplastic size scale

Over 80% of microplastics are produced on land, while less than 20% come from the sea. Due to their lightweight, indestructible nature, and ability to float, microplastics can travel great distances globally (Browne et al., 2011; Karbalaei et al., 2018). Most plastics pollute the marine environment from land-based sources, fishing, aquaculture, and coastal tourism (Browne et al., 2015; Thushari & Senevirathna, 2020). It is estimated that over

INTRODUCTION

800 million tons of plastics in the sea originated from land (Jambeck et al., 2015). Micro- and nanoplastics are so small that wastewater treatment processes cannot filter them out, leading to their presence in rivers, oceans, and freshwater supplies (Vance et al., 2015). Additionally, these plastics are found in soil and can enter water systems through natural erosion (Horton et al., 2017). According to the United Nations Environment Program, 275 million tons of plastic waste were produced in 2010, with an estimated 4.8–12.7 million tons entering water systems (*Microplastic Contamination in Aquatic Environments*, 2018). Micro- and nanoplastics come from both primary and secondary sources. Primary sources are those intentionally created for consumer and industrial uses, such as exfoliants in cleansers, cosmetics, drug delivery particles in medicines, and industrial air blasting. Secondary sources are macroplastic products that break down into smaller particles, occurring both on land and in aquatic environments (Karbalaei et al., 2018). Plastics break down into micro- and nanoplastics through biodegradation and non-biodegradation processes. Non-biodegradation includes thermal, physical, photodegradation, thermo-oxidative degradation, and hydrolysis. These processes break down plastics into smaller particles, altering their properties and increasing their surface area, which enhances interactions with microorganisms. Biodegradation involves environmental bacteria and microorganisms that produce enzymes to break down plastics into nano-sized particles. A single gram of microplastic can yield billions of nanoplastic particles. Plastic waste fragments grow faster on coasts due to higher exposure to UV radiation, higher temperatures, and the presence of salt, compared to the ocean. High saline content and microorganisms in marine areas also accelerate plastic degradation (Yee et al., 2021). These micro and nano-sized plastic particles can enter the human body through three main routes including ingestion, inhalation, and dermal contact (Figure 5). Many human tissues and fluids have been reported to host microplastics, including lung tissue, blood, the placenta, breast milk, and atheromas. More recent studies have confirmed their presence in the lung tissues and human blood samples. The presence of microplastics has been identified in placentas and, even more problematically, in breast milk. Detection in atheromas might suggest some risk associated with cardiovascular health. These findings give rise to formidable concerns about health effects associated

with the presence of microplastics in human biological systems(Cheng et al., 2024; Enyoh et al., 2023; Hutter et al., 2024; Osman et al., 2023)**.**

As previously mentioned (Andrady, 2017), plastic polymers are categorized based on their physiochemical and thermal features in various types including, PS, PET, PVC, PP, PU, and phenol-formaldehyde (PF). Each plastic polymer has its features and application (Figure 4). Thus, PE is composed of repeating units of ethylene (C_2H_4), making it a simple polymer with a high degree of crystallinity. It is widely used in packaging, such as plastic bags and bottles, as well as in containers and household goods. Environmentally, PE is highly persistent and constitutes about 34.5% of global plastic production. While generally considered safe for food contact, concerns arise from additives and potential chemical leaching. PP is made from the polymerization of propylene (C_3H_6). It boasts a high melting point and resistance to many chemical solvents, making it suitable for packaging, automotive parts, textiles, and reusable containers. PP is also persistent in the environment, accounting for about 19.3% of global plastic production. Similar to PE, PP is considered safe, but additives can pose health risks. PVC consists of vinyl chloride monomers (C_2H_3Cl) and can be made flexible or rigid by adding plasticizers. It is commonly used in construction materials like pipes and windows, medical devices, and packaging. PVC poses significant environmental hazards due to its chlorine content and makes up about 10.2% of global plastic production. Health risks associated with PVC include the release of harmful chemicals like dioxins during production and disposal. PS is derived from styrene monomers (C_8H_8) and can be either solid or foamed. It is used in packaging, disposable cups, insulation, and consumer electronics. PS is a major component of marine litter and is difficult to recycle, representing about 6.1% of global plastic production. Styrene, a component of PS, is a suspected carcinogen, and exposure can occur through food contact materials. PET is composed of terephthalic acid and ethylene glycol, forming a polyester with high strength and clarity. It is commonly used in beverage bottles, food packaging, and synthetic fibers. While PET is widely recycled, it still contributes to environmental pollution and represents about 7.4% of global plastic production. Generally considered safe, concerns exist over antimony leaching from PET bottles (Law et al., 2024 Arias-Andres and Rojas-Jimenez, 2022).

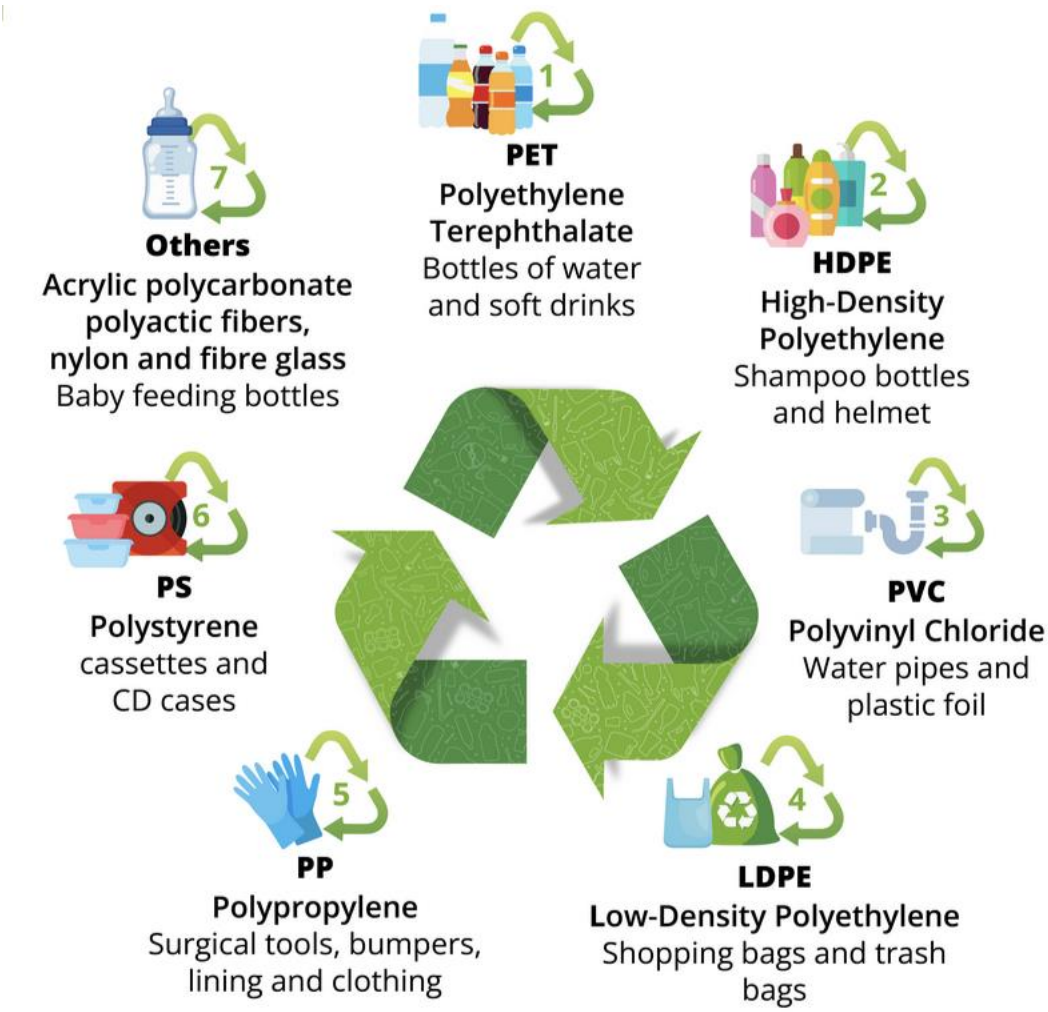


Figure 4. Different types of plastic polymers and their application.

The global production of plastics has reached over 368 million metric tons annually. The dispersity of these plastics in the environment varies, with PE and PP being the most prevalent due to their extensive use in packaging. Plastics are a major component of marine debris, with MPLs being particularly concerning. Studies estimate that 8 million metric tons of plastic enter the oceans each year. Recycling rates vary by polymer type, with PET and HDPE (High-Density Polyethylene) having higher recycling rates compared to other plastics. In addition, plastics can release harmful chemicals during their lifecycle, including production, use, and disposal. These chemicals include additives like plasticizers, flame retardants, and stabilizers. Some plastic additives, such as bisphenol

INTRODUCTION

A (BPA) and phthalates, are known endocrine disruptors, which can interfere with hormone function. Plastics are indispensable in modern society due to their versatility and wide range of applications. However, their environmental persistence and potential health impacts necessitate careful management and regulation. Increasing recycling rates, reducing the use of harmful additives, and developing biodegradable alternatives are crucial steps toward mitigating the negative effects of plastic pollution (Alabi et al., 2019; Whitfield et al., 2019; Arias-Andres and Rojas-Jimenez, 2022; Law et al., 2024).

Among the various types of plastic polymers, PS is commonly used in industry and daily human activities, especially in food packaging (Lee et al., 2024). PS is an aromatic polymer derived from the polymerization of styrene monomers, which are produced from ethylene and benzene. The large-scale production of PS involves the catalytic dehydrogenation of ethylbenzene to form styrene monomers. As a thermoplastic polymer, PS is known for its high translucency, durability, and ease of dyeing. It is commonly used in the manufacture of CDs, toys, and toothbrushes. Additionally, PS is utilized to produce styrofoam, a material with limited elasticity that is expanded or melt-formed by rapidly heating PS pellets with a foaming agent. Styrofoam is widely employed in the production of food containers, such as trays, plates, and cups, as well as for food storage, transport, and various packing products, toys, clips, and office supplies (Kik et al., 2020).

PET is the most common thermoplastic polymer used in the production of clothes and packaging for food and beverages. It boasts advantages such as being lightweight, having high tensile strength, being transparent, and providing an effective gas barrier (Dhaka et al., 2022). By 2022, the worldwide production of PET is projected to hit 87.16 million metric tons. PET is made up of repeating units of ($C_{10}H_8O_4$) and it is widely recycled and identified by the resin identification code (RIC) number 1 (♺). According to the National Association for PET Container Resources (NAPCOR), PET items are derived from terephthalic acid (or dimethyl terephthalate) and mono ethylene glycol, with these components making up at least 90% of the mass of monomers used to create the polymer (NAPCOR, 2020). Unfortunately, a significant portion of PET ends up as plastic waste in the natural environment after just one use (Urbanek et al., 2021).

Like polystyrene, PET microplastics have been found in the ocean, indicating that this material can break down and reduce in size when subjected to environmental conditions (Suaria et al., 2016). To better understand the impacts of environmentally derived PETNPLs on humans, true-to-life PET nanoplastics, created through laser ablation, interact with waterborne pollutants to form nanoclusters. These nanoclusters impact cellular metabolism, indicating potential long-term risks (Magrì et al., 2021a). One of the main uses of this polymer is in beverage containers like bottled water, which generates the question of how toxic they are when they can accumulate in the environment and reach the human body. The presence of PETNPLs less than 5 µm in drinking, water and beverage products was confirmed; among them, smaller particles are more abundant in tap and mineral water (Mortensen et al., 2021).

1.3. Human exposure to micro- and nanoplastics

Their universal presence in different environmental matrices has made human exposure to them a really concerning issue. Different pathways of entry into the human body by MNPLs are through ingestion, inhalation, and dermal exposure. Some recent studies underlined their possible health implications by suggesting that the particles are well present in different human fluids and tissues. MNPLs are ingested into the human body through contaminated food and beverages. Hwang et al. (2022) determined microplastics in a variety of foods such as seafood, salt, and bottled water, indicating potential dietary intake of microplastics. Other studies by Cunha et al. (2023) have pointed out that many foods commonly consumed by the general public do indeed contain microplastics, showing just how pervasive these contaminants have become in our diets. Inhalation is another route of considerable exposure, especially to those people living in urban areas or near industrialized areas. For example, Bermudez et al. (2022) reported the presence of microplastic elements in human lung tissues and raised concerns with regards to respiratory health and also the systemic absorption of such elements. Similarly, Leslie et al. (2022) found the incidence of microplastics in human blood samples, which may indicate that these particles enter the circulatory system and further spread to other body

parts through the blood. Furthermore, microplastics have been found in human placental tissues (Ragusa et al., 2021) and in breast milk (Alfonso et al., 2021). These statements show that the capabilities of MNPLs are not only going to affect adults but also the sensitive groups: fetuses and babies. The consequences of these exposures remain under investigation, but detecting microplastics in such vital biologic systems brings up important questions about its possible health effects.

1.3.1. Ingestion

The increasing amount of plastic waste has led to micro- and nanoplastics infiltrating the food chain, posing a significant health risk. These tiny particles are found everywhere, from water bodies to land, making their presence in food products almost certain (Yee et al., 2021). Research indicates that micro- and nanoplastics enter our food chain through various means; animals ingesting them in nature (Santillo et al., 2017), contamination during food processing (Karami et al., 2017), and leaching from plastic packaging (Mason et al., 2018). These particles have been detected in a variety of foods and the use of Fourier-transform infrared spectroscopy (FTIR) has shown that microplastics are present in tap, bottled, and spring waters (Kosuth et al., 2018). On average, a person consumes between 39,000 and 52,000 microplastic particles annually, with the total varying by age and gender. When inhalation is considered, this number increases to between 74,000 and 121,000 particles per year. People who drink only bottled water may ingest an additional 90,000 particles annually compared to those who drink tap water, who consume about 4,000 extra particles (Cox et al., 2019). These findings highlight that the human food chain is a major source of microplastic intake and research showed the ingestion as the major rout of entry for nanoplastics (Sun & Wang, 2023) .

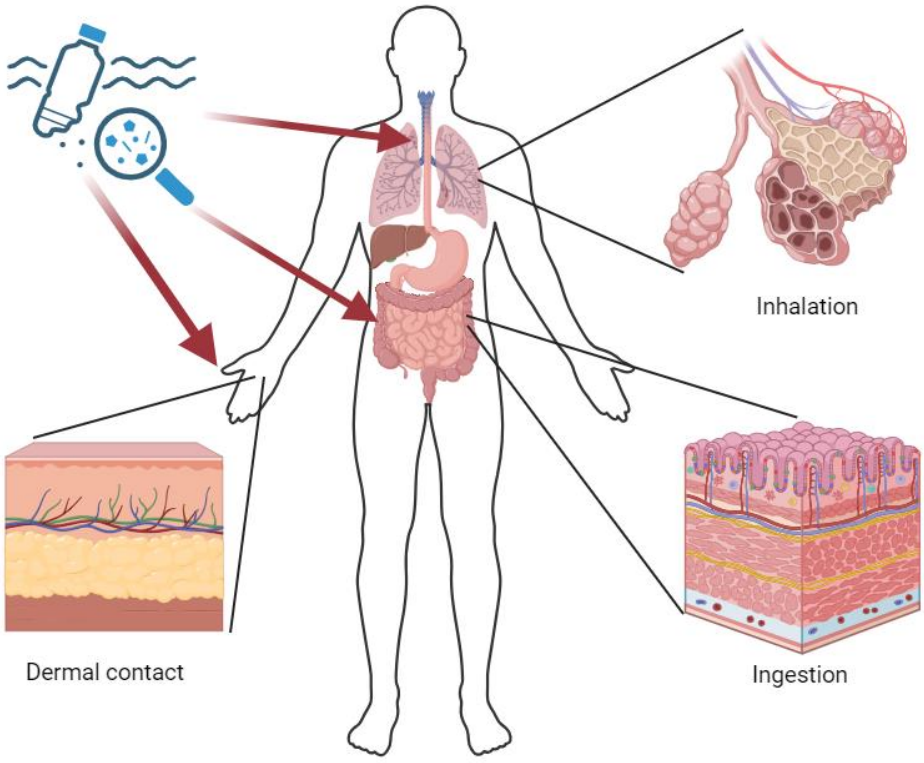


Figure 5. Three main routes of nanoplastic entry, including inhalation, ingestion, and dermal contact.

To meet the global demand of humans, the food production industry has grown significantly during the last decades. Considering the overall categorization of food, around 19% of the global population uses seafood as their chief source of animal protein which shows the high dependence of humans to the ocean's derived food (Beaumont et al., 2019). For instance, mussels as filter feeders act as vectors for micro and nanoplastic translocation while humans are using them as a source of protein in their daily dietary plan. The same criteria apply to other species like fish, prawns, and squid with this notion that most of the plastics are accumulated in inedible tissues in comparison to edible tissue like the digestive tract (Ramsperger et al., 2023). These plastics are also ingested through contaminated food and water (Karbery et. al., 2018). Different studies showed that nanoplastics can cross the gastrointestinal tract and reach the circulatory system (Lett et al., 2021).

1.3.2. Inhalation

MNPLs can enter the human body through inhalation as an important route (Prata et al., 2020; Rahman et al., 2021). Airborne particles from urban dust, synthetic textiles, and rubber tires degradation products can be inhaled (Prata, 2018). However, indoor air exposure poses a greater risk for inhaling MNPLs. Indoor concentrations are significantly higher than outdoor levels and European citizens spend most of their time indoors, which is relevant for risk estimations. Methods for assessing airborne MNPLs include direct filtering, passive sampling, and mannikin-based approaches. Plastic particles can reach and accumulate in the pulmonary and nasal system in humans based on their size and shape like the plastic fibers that upon reaching the pulmonary system are difficult to remove by mucociliary escalator while the larger particles can be removed by this defense system (Stapleton, 2021).

Inhalation is one of the routes for MNPLs to enter the human body especially at the alveoli, showing an abundance rate of between 0.56 to 1.42 particle/g. These findings are even though MNPLs are usually removed by nasal hair blockage, mucus cilia adhesion, or macrophage phagocytosis (Feng et al., 2023).

1.3.3. Dermal contact

Despite dermal contact being considered the least important pathway for plastic particle entry, several shreds of evidence revealed that microbeads and microfibers in personal care products can cross the skin barriers and reach the human body (Ali et al., 2024a). Although the skin usually blocks particles, they can still enter through wounds, sweat glands, or hair follicles. Due to legislative pressure since 2018 large quantities of plastics in personal care products were substituted with natural substances (Chaudhari et al., 2024). Still, this replacement could not alleviate the concern about the presence of MNPLs in personal skin care products because particles less than 100 nm still are being used in this kind of product (Feng et al., 2023). While the likelihood of MPLs absorption through the skin is generally low due to the stratum corneum’s barrier, it is crucial to consider the potential penetration of nanoplastic particles smaller than 100 nm. Workers in plastic-related industries and individuals engaging in leisure activities near microplastic pollution sites should be aware of this possibility, despite the lack of definitive studies on

the matter. Skin irritation and other health issues may result from direct contact with microplastics (Revel et al., 2018).

1.3.4. Nanoplastics translocation through human barriers

Considering the small size of the environmental NPLs, their ability to enter the human body is inevitable. The rising questions and concerns about the possible outcomes of this exposure are due to the ability of these tiny particles to translocate through human biological barriers (Figure 6). There are significant shreds of evidence showing the ability of nanoplastics to cross human barriers in the gastrointestinal tract, pulmonary system, and other organs when they enter by ingestion. Barrier translocation studies started by focusing on intestine since is the major and primary route of exposure to nanomaterials and nanoplastic. Domenech et al. (2020) evaluated the barrier-crossing ability of PSNPLs in humans using Caco-2, HT-29, and Raji-B cell lines to simulate *in vitro* the human intestinal barrier and its associated lymphoid system and the membrane integrity analysis showed the translocation of the polystyrene nanoparticles through this barrier (Domenech et al., 2020). Since ingestion is not the only route for nanoplastic entry, other biological barriers like the pulmonary one must be considered when inhalation route is considered. Regarding this issue, (Annangi, Villacorta, Vela, et al., 2023) evaluated the potential toxicity and barrier integrity of PS- and PETNPLs in human nasal epithelial cells as a matter of fact that they are the initial barriers in the process of inhalation of nanoplastics. That study revealed the ability of NPLs with different polymers to cross the barriers and reach the circulatory system. In addition, other *in vivo* studies also revealed that NPLs are capable of crossing biological barriers. For instance, two separate studies detected the translocation of MNPLs through the placental barriers in rodents and, as a result, they ended up in fetal tissues (Fournier et al., 2020; Cary et al., 2023) . on the other hand (Alaraby et al., 2022a) showed the translocation of PETNPLs through the intestinal tract in Drosophila larvae.

The outcome of barrier crossing by NPLs is reaching the circulatory system and being exposed to blood circulation, which may result in the translocation of plastic particles into secondary organs. In blood, the different cell types would be the target of MNPLs. To determine the effects of MNPLs on such types of cells, different human hematopoietic

cell lines were exposed to PS NPLs resulting in genotoxic damage in such cell models (Rubio et al., 2020a).

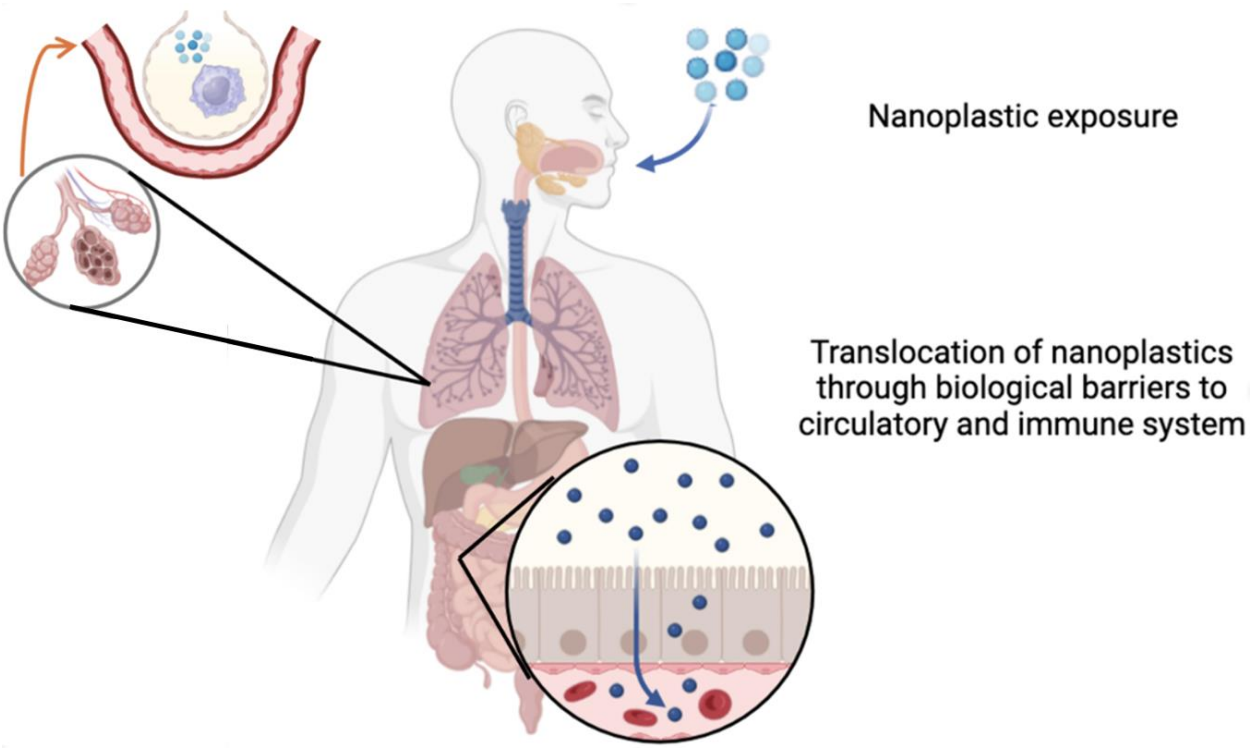


Figure 6. Schematic overview of nanoplastic translocation through human barriers.

1.3.5. Factors affecting nanoplastic translocation

The translocation of NPLs within the human body is influenced by several factors, including particle size, surface charge, chemical composition, shape, and exposure routes. The size of NPLs plays a crucial role in their ability to penetrate biological barriers. Smaller particles can more easily cross cell membranes and enter systemic circulation. For instance, particles less than 100 nm can translocate across the gastrointestinal tract and respiratory epithelium more efficiently than larger particles(Llorca & Farré, 2021a). Additionally, the surface charge of nanoplastics affects their interaction with cellular membranes. Positively charged particles tend to have a higher affinity for negatively charged cell membranes, facilitating their uptake and translocation (Xie et al., 2023).

The chemical composition of nanoplastics also significantly impacts their translocation. Hydrophobic nanoplastics, such as those made from PP and PVC, can more easily traverse the mucus layer in the gastrointestinal tract, enhancing their absorption (Llorca & Farré, 2021a). Moreover, the shape of nanoplastics influences their movement within the body. Fiber-shaped NPLs, for example, can translocate more efficiently from the tip, compared to spherical particles (Li et al., 2022a).

Therefore, understanding the factors affecting NPLs translocation in the human body is essential for assessing their potential health risks. Particle size, surface charge, chemical composition, shape, and exposure routes all play critical roles in determining the extent and efficiency of their translocation. Continued research in this area is necessary to develop effective strategies for mitigating the health impacts of NPLs exposure.

1.4. Potential impacts of MNPLs on human health

The spreading concern of possible impacts on human health, because of the pervasive presence in the environment of microplastics and nanoplastics, is discussed, along with the multiple routes of exposure. Research studies indicate that MNPLs cause adverse effects to various organ systems, leading to serious health risks. Exposure is highest through ingestion, inhalation, and, to a lesser extent, by dermal contact. MNPLs ingested through contaminated food and water may result in the disturbance of the GI tract due to oxidative stress and inflammation (Otorkpa & Otorkpa, 2024; Stoian, 2024). In addition, other elements may be inhaled into the respiratory system and fall deep inside the lungs, hence potentially contributing to the development of respiratory ailments such as asthma and reduced pulmonary performance. According to (Nazeer et al., 2024; Vasse & Melgert, 2024), in this way, exposure by inhalation can thus involve the potential for respiratory diseases, but less is known about dermal uptake, which is considered especially relevant in occupational environments. Other health effects include disruption of intestinal homeostasis, resulting in dysbiosis and metabolic disorders, while respiratory effects include inflammation and exacerbation of pre-existing conditions. Cardiovascular effects also have been linked to increased morbidity and mortality rates. In the light of such findings, there is still considerable lack in grasping the holistic health consequences of

MNPLs, since most research target particular types of MNPLs while leaving other types unconsidered. Future research should be recommended to head toward comprehensive exposure and health outcome assessments that might guide public health strategies. Other scholars have also emphasized that health impact studies of MNPL are still at their infancy and have called for extensive epidemiological studies to establish any cause-and-effect relationship or long-term effects since the sampling into risks of MNPL is complex and an entity of ongoing investigation (Ali et al., 2024b).

1.4.1. Polystyrene nanoplastic (PSNPLs)

Polystyrene nanoplastics have aroused wide concern due to their possible health effects. Such particles may enter the human body through respiration, ingestion, and skin. After entering the body, PSNPLs can be transported to several organs and tissues, further causing a series of adverse health effects. One of the major problems with PSNPLs is that they provoke oxidative stress. It has been recorded in numerous studies that PSNPL exposure can lead to the formation of reactive oxygen species, which may cause the degradation of cellular molecules like DNA, proteins, and lipids. This type of oxidative stress could ultimately lead to inflammation, cell death, and even promote the formation of chronic diseases such as cancer. Other than that, the significant impacts of PSNPLs on health include the potential for impact on the immune system. PSNPLs have been shown to modulate immune responses toward either immunosuppression or immune hyperactivation. This dysregulation of the immune system will make an individual more susceptible to infections and other diseases. Furthermrpe, PSNPLs have been found to affect the nervous system. Such studies show that maternal exposure to PSNPLs causes neurotoxicity in offspring, marked by anxiety and depression, including abnormalities in social behaviors. This would mean that PSNPLs can affect longer-term changes in brain development and function. Besides these, PSNPLs can cause physical damage to tissues. For example, exposure to PSNPLs is associated with gut damage, locomotor dysfunction, and epigenetic changes. These physical effects can also further contribute

to the health risk of PSNPLs (Alaraby et al., 2022b; Annangi, Villacorta, López-Mesas, et al., 2023; J. Chen et al., 2024; Preda et al., 2024).

1.4.2. Polyethylene terephthalate nanoplastics(PETNPLs)

Recent studies have pointed out the systemic toxicological effects of polyethylene terephthalate nanoplastics on liver function and cellular metabolism. It was indicated that PETNPLs could affect a wide range of liver-targeting metabolites, especially in detoxification pathways and oxidative stress responses. Such disturbance hinders the normal role of the liver and is likely to result in interference with the toxin-clearing capability and the control of cellular homeostasis. Another finding in the study concerns mitochondrial integrity. Mitochondrial membrane stability is an important feature for maintaining energy production and cellular function, disrupted by PETNPLs. This shows up as increased polar head groups in phospholipids, indicating membrane stress and breakdown. The impaired membrane compromises mitochondrial efficiency, leading to cellular energy deficits and increased oxidative stress, which is further likely causing cellular structural damage. Moreover, exposure to PETNPLs introduces broad perturbations in metabolites of interconnected pathways of cellular bioenergetics. The metabolome cascade changes within key energy-producing processes such as glycolysis, the tricarboxylic acid cycle, and oxidative phosphorylation. These alterations in metabolism not only diminish the availability of energy within cells but may also promote changed metabolic states that predispose to disease. Together, these findings contribute to an integrated understanding of PETNPLs toxicity, representing the first system-level examination of its harmful effects, particularly in early developmental stages, as done in intact larvae studies. This therefore calls for more research into further determinations of the toxicity of PETNPLs across systems and life stages (Bashirova et al., 2023) . Polystyrene nanoplastics (PETNPLs) have been evaluated for their potential hazards, particularly in primary human nasal epithelial cells, which serve as a target for inhalation exposure. Studies have demonstrated that PETNPLs can be internalized by these cells and induce the production of reactive oxygen species (ROS), leading to oxidative stress. Additionally, exposure to PETNPLs has been shown to cause a loss of mitochondrial

membrane potential, which is crucial for cellular energy production and function. This disruption further affects cellular health by inducing changes in the autophagy pathway, a mechanism essential for maintaining cellular homeostasis. These findings underscore the potential health risks posed by PETNPLs, highlighting their impact on respiratory cells and cellular processes(Annangi, Villacorta, Vela, et al., 2023). Since they are environmentally dispersed, they can reach the human body and can considerably affect human health; for instance, toxic effects of PETNPLs on human lung epithelial cells have been shown as induction of cellular stress and even DNA damage (Alzaben et al., 2023)

1.4.3. Different types of nanoplastics for biological studies

There are considerable differences between terminologies of nanoplastics and nanoparticles and knowing them not only affect the criteria of the research but also improve the main hypothesis about the environmental toxic effects of plastic particles. Engineered nanoparticles are intentionally designed and manufactured particles with at least one dimension between 1 and 100 nm. These particles are created to exploit their unique physical and chemical properties for studying the health-related effects. The small size of engineered nanoparticles allows for a high surface area to volume ratio, leading to enhanced reactivity, strength, and electrical properties (Jarvie, 2024).

On the other hand, NPLs, defined by their extremely small size, typically between 1 and 1,000 nm. Unlike engineered nanoparticles, secondary nanoplastics are not intentionally produced but are formed through the degradation of larger plastic materials. This degradation can occur due to environmental factors such as sunlight, mechanical wear, and chemical processes (Mohan Qin, 2024). NPLs are found in various environments, including oceans, soil, and even the air. Their small size allows them to be easily transported over long distances and penetrate in organisms reaching biological tissues, raising concerns about their potential impact on human health and ecosystems. Studies have detected nanoplastics in human blood, liver, lung cells, and reproductive tissues (Bikiaris et al., 2024). NPLs originate from the breakdown of everyday plastic products such as packaging, clothing, and personal care items. In the environment, the degradation process can be accelerated by factors like UV radiation and physical

abrasion. As plastics degrade, they fragment into smaller and smaller pieces, eventually reaching the nanoscale (Vicki Contie, 2024).

Considering the origine of the mentioned materials, in this study we aimed to use NPLs as test materials since engineered nanoparticles are intentionally designed and manufactured for specific applications, whereas environmental NPLs are incidental byproducts of plastic degradation. This intentionality in production leads to significant differences in their properties and applications (Auffan et al., 2009).

The ability of nanoplastics to penetrate biological tissues and accumulate in organisms poses significant risks, whereas engineered nanoparticles are designed to minimize such risks in their intended applications (Gigault et al., 2021). Thus, engineered nanoparticles and environmental secondary NPL represent two distinct categories of nanoscale materials with different origins, characteristics, and implications.

Among these types of NPLs for biological studies, there is a form called True-to-life NPLs that are accounting for those made from real environmental plastic material; thus, they provide a critical model to understand the realistic toxicity of environmental NPLs. Recent studies have emphasized the different biological interactions which these NPLs possess compared to synthetic NPLs. For instance, a true-to-life PET nanoplastics have been found to induce oxidative stress and disruption in gene expression in model organisms like Drosophila melanogaster, thus providing an accurate estimation of the health impacts and cellular interaction more so than the synthetic nanoplastics. More realistic nanoplastics, or NPLs, created through environmental degradation, can model their potential toxicity more accurately than synthetic nanoplastics. Thus, NPLs demonstrated cell penetration, induction of oxidative stress, inflammation, and even DNA damage; hence, these particles are more relevant in terms of real health risks from a standpoint of nanoplastic pollution (Alaraby et al., 2023) (Figure 7).

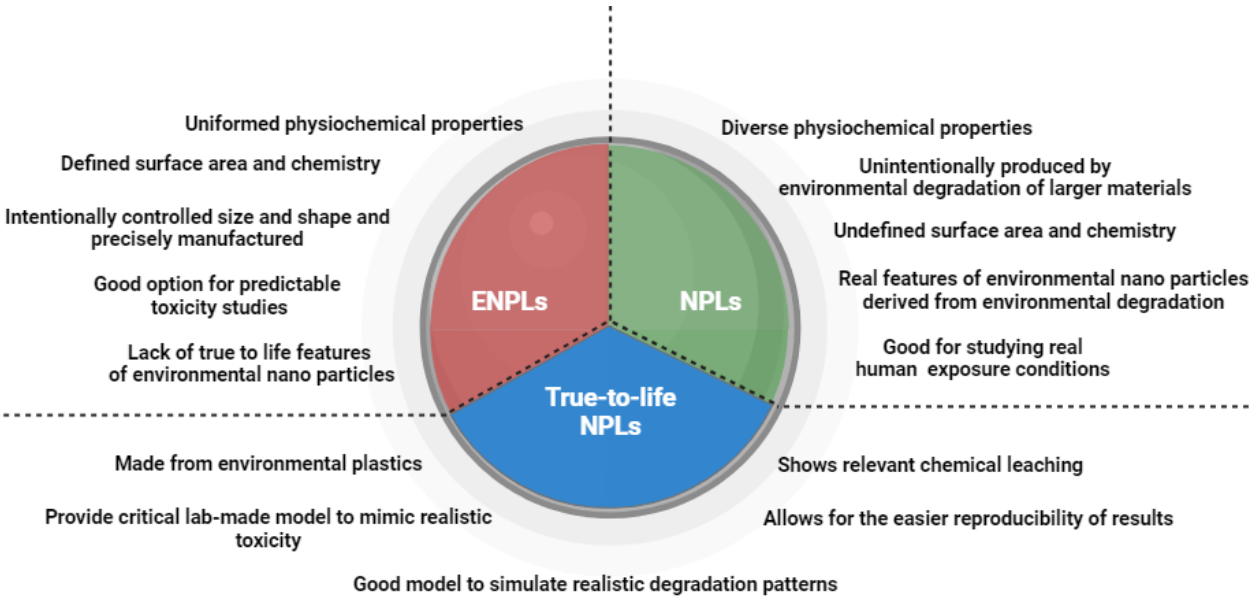


Figure 7. Engineered nanomaterials (ENMLs), True-to-life NPLs, and nanoplastics (NPLs) comparisons.

1.4.4. Impact of MNPLs on blood system

As previously described, NPLs can cross different barriers, reaching to blood system that enables them to translocate in other organs. Therefore, various published studies assessed human samples including, colon, kidney, blood, urine, vein, saliva, breast milk, liver, and even placenta to detect the toxic effect related to these particles (Bai et al., 2024). Among the studies focused on the toxicity of NPLs based on their polymer type in human samples PET and PS nanoplastics show a common route for delivery of environmental nanoplastics into the human body which is blood and other closed body fluids (Guan et al., 2023); the discovery and quantification of plastic polymers in human blood was studied (Leslie et al., 2022) revealing valuable and significant information about the presence of different plastic polymers including PS and PET in human blood after analyzing the blood of 22 healthy donors. Another study investigated the presence of NPLs in human peripheral blood by employing fluorescent techniques and nano cytometry, along with staining using the lipophilic dye Nile Red, and they demonstrated that NPLs can be precisely detected through flow cytometry (Salvia et al., 2023). In addition to the natural blood flow that enables NPLs to translocate throughout the body,

their direct contact with human blood cells is a great concern. Exposure to PSNPLs in human lymphocytes led to a notable rise in micronuclei (MN) formation and cytostasis, along with a dose-dependent decrease in the nuclear division index (NDI). These changes suggest that PSNP exposure causes oxidative stress, resulting in cytotoxicity, DNA damage, and genomic instability (Sarma et al., 2022). Also, in a very recent ex vivo study, the toxicity of five different plastic polymers were evaluated in 8 human blood samples showing, NPLs internalization, ROS generation, cytokine release, hemolysis, and platelet functionality in whole blood cells (Arribas Arranz et al., 2024).

1.5. Nanoplastics and Immunotoxicity

1.5.1. Overview of immune system mechanism

The immune system is designed to protect against invading agents such as pathogens and environmental pollutants-including micro and nanoplastics by two mechanisms including innate and adaptive immunity. Innate immunity is described as the body's immediate nonspecific response via barriers like the skin and mucous membranes, and it involves various immune cells such as monocytes and macrophages. These recognize common characteristics of pathogens or foreign particles and destroy them through an active response against them. Adaptive immunity takes a little longer to set in but is very specific; this involves lymphocytes, which are primarily T and B cells. These lymphocytes recognize antigens and develop focused attacks. The lymphocytes can act either directly through the killing ability of T cells by lysing infected cells or indirectly through the production of antibodies by B cells, which neutralize attackers. Another type of WBC, monocytes, can develop into macrophages. These are the major participants of innate immunity; these cells are phagocytic, taking up and digesting infectious agents and dead cells. Further, these macrophages polarize into different states depending upon the signal received from the outside environment (Murphy & Casey Weaver., 2016) .

1.5.2. Immune response toward MNPLS exposure

As mentioned in previous parts, due to the permeability biological barriers toward NPLs, they can cross the barriers and reach several defensive systems in the body which generally prevent environmental contaminants and pathogens to affect the human body

upon exposure. While reacting against exposure to MNPL, lymphocytes may release pro-inflammatory cytokines, exacerbating inflammation in the tissues with the potential for tissue damage. Thus, MNPLs polarize macrophages toward the M1 state, which may cause chronic inflammation and eventually induce tissue injury. Such a dual response underlines how MNPLs can affect both immune branches, promoting inflammation and cytotoxicity in immune cells, which seriously affects human health. When NPLs cross barriers, they will face this line of defense including several immune cells and this exposure may affect the normal function of these cells and cause inflammation and toxicity (Wolff et al., 2023a; Hirt & Body-Malapel, 2020a). Due to the high importance of the immune system for body hemostasis, there is a global concern about the immune interfering capacity of NPLs that increases the rate of different biological cascades in response to inflammation and cytotoxicity.

MNPLs have emerged as significant environmental pollutants with potential implications for human health. These particles, due to their small size, can penetrate epithelial barriers and interact with various biological systems. One critical aspect of their impact is on the immune system, particularly through the activation of the NLRP3 inflammasome. That inflammasome is a vital component of the innate immune system, responsible for detecting pathogen- and damage-associated molecular patterns. Activation of this inflammasome leads to the production of pro-inflammatory cytokines, such as IL-1β and IL-18, which are crucial for initiating and sustaining inflammatory responses. A recent paper “has explored how MNPLs can activate the NLRP3 inflammasome, thereby triggering inflammatory pathways (Alijagic et al., 2023a). A schematic overview is presented in (Figure 8).

1.5.3. *In vivo* studies

The immunotoxicity of MNPLs has been studied in various aquatic species and mice. In fathead minnow, exposure to PS and PMMA MPLs increased myeloperoxidase activity and neutrophil extracellular trap release, while reducing oxidative stress. PMMA MPLs disturbed neutrophil function in fathead minnow, but PS microbeads showed no

significant immunotoxicity in trout. PE MPLs impaired the complement system in carp, and PVC MPLs affected phagocytic capacity and respiratory burst in fish. In zebrafish, high-density PE and PS particles decreased liver immune gene expression. In mice, PE MPLs altered serum cytokine levels, reduced regulatory T cells, and increased Th17 cells. In addition, cross-generational effects included elevated neutrophil counts and altered spleen lymphocytes. In catsharks, macroplastics exposure upregulated immune gene expression in the spleen. These findings underscore the need for further immunotoxicity research on species more closely related to humans (Hirt & Body-Malapel, 2020a). The importance of immune related effects toward NPLs are mentioned in previous studies. At the same time, still there are several gaps in different aspects of this line of study like the cell type to be used. These NPLs physiochemical effects raise the necessity of assessing such a hypothesis in deeper lines. (Greven et al., 2016) observed dose-dependent myeloperoxidase activity and neutrophil extracellular trap release in fathead minnow when exposed to PS NPLs of 41 nm. Considering similar studies and analyzing the outcomes, we can determine how the effects in different study models, polymer type, and experimental conditions affect the immunotoxicity of NPLs.

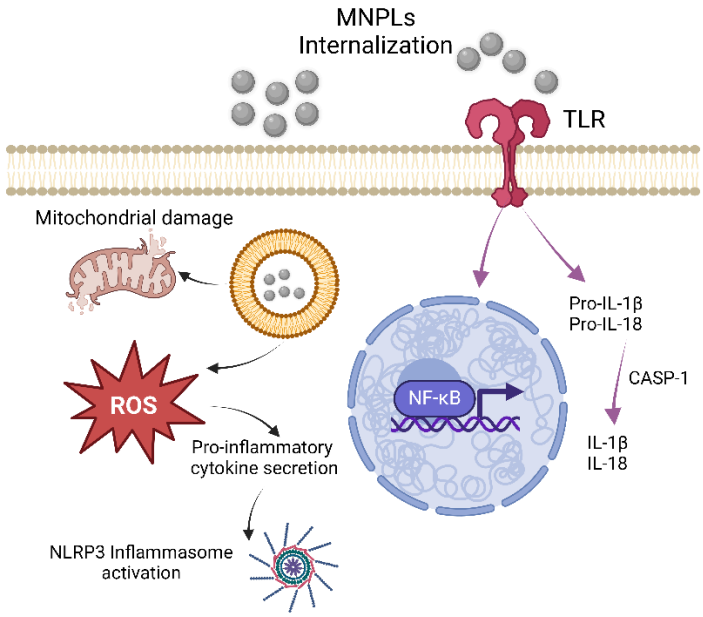


Figure 8. Schematic overview of inflammation derived from MNPLs interaction.

1.5.4. *In vitro* studies

Recent *in vitro* studies have highlighted the potential immunotoxic effects of NPLs, particularly PSNPL, on human cells. One study assessed the cytotoxic and genotoxic potential of PSNPLs on human peripheral lymphocytes using chromosomal aberration and cytokinesis-block micronucleus assays. The results indicated that high concentrations of PSNPLs could induce significant chromosomal damage and genotoxic effects (Sarma et al., 2022). Another study utilized a triple culture model to simulate the acute toxicological effects of plastic particles in both healthy and inflamed human intestinal cells. This model revealed that PSNPLs could exacerbate inflammatory responses and disrupt cellular integrity, suggesting a potential risk to human health (Xie et al., 2023). Additionally, other studies have shown that NPLs can interact with various cellular components, leading to oxidative stress and immune cell toxicity. For instance, exposure to NPLs can impair oxidative and inflammatory balance in intestinal cells, disrupt gut epithelial permeability, and cause dysbiosis, which is an imbalance in the gut microbiota (Hirt & Body-Malapel, 2020a). These findings underscore the importance of further investigating the long-term health implications of NPLs exposure, as their widespread presence in the environment could pose significant risks to human health.

1.5.5. The THP-1 cell line

The THP-1 cell line, derived from a 1-year-old male with acute monocytic leukemia, is a widely utilized human monocytic cell line in biomedical research. Established by Tsuchiya et al. (1980), THP-1 cells exhibit monocyte-like characteristics, including the expression of surface markers such as CD11b, CD14, and HLA-DR. They can differentiate into macrophage-like cells upon stimulation with agents like PMA, IFN-γ, or LPS, which alters their morphology and adherence properties while upregulating macrophage-specific markers. This makes THP-1 cells a valuable model for studying monocyte and macrophage biology, immune responses, drug screening, and toxicology. The human origin and reproducibility of THP-1 cells make them particularly relevant for immunological studies, where they are used to investigate cytokine production, phagocytosis, and inflammation. Additionally, they serve as a model for assessing cytotoxicity, genotoxicity,

and oxidative stress in toxicology and environmental studies, and for testing vaccine efficacy (Auwerx, 1991; Daigneault et al., 2010; Park et al., 2007; Tsuchiya et al., 1980).

Different studies have demonstrated that exposure to nanoplastics can lead to the activation of THP-1 cells, resulting in the release of pro-inflammatory cytokines and the generation of reactive oxygen species (ROS), which indicate cellular stress and potential oxidative damage (Schirinzi et al., 2017; Wu et al., 2019a). This model is particularly valuable for understanding the cellular mechanisms underlying plastic-induced inflammation and for evaluating the potential health risks associated with plastic pollution in humans (Vandermeersch et al., 2015). THP-1 cells are mononuclear and exhibit similar morphological and functional properties to primary human monocytes. Upon PMA (Phorbol 12-myristate 13-acetate) stimulation, THP-1 cells differentiate into adherent macrophage-like cells that display enhanced phagocytic activity, cytokine production, and expression of macrophage-specific markers such as CD68 (Polasky et al., 2022). This differentiation process is associated with significant changes in gene expression profiles, highlighting the cell line's utility in studying macrophage biology.

THP-1 cells are often employed to investigate inflammatory responses. They respond to various stimuli, including bacterial lipopolysaccharides (LPS), with the activation of key signaling pathways such as NF-κB and MAPK (Mitogen-Activated Protein Kinases) (Kawasaki & Kawai, 2014). These pathways are critical in mediating the inflammatory response, and the THP-1 cell line serves as a valuable tool for dissecting the molecular mechanisms underlying inflammation and immune responses.

1.5.6. The TK6 cell line

The TK6 cell line, derived from the lymphoblasts of a 5-year-old male with chronic myeloid leukemia, is a widely utilized human lymphoblastoid cell line in genetic toxicology and biomedical research. TK6 cells are characterized by a stable near-diploid karyotype and the presence of a heterozygous thymidine kinase (TK1) gene, which is commonly used as a marker in mutagenicity assays (Saleh et al., 2015). Upon exposure to mutagens, TK6 cells can undergo mutations at the TK1 locus, leading to the loss of thymidine kinase activity, a feature that is exploited in the thymidine kinase gene mutation assay to assess mutagenic potential. TK6 cells are particularly valuable for studying DNA damage and

repair mechanisms, as they possess functional p53 and are capable of undergoing p53-mediated cell cycle arrest and apoptosis in response to genotoxic stress. This makes them an ideal model for investigating the molecular pathways involved in cellular responses to DNA damage. In addition, TK6 cells are employed in cytotoxicity assays, where they are used to evaluate the potential toxic effects of new drugs, chemicals, and environmental pollutants. (Saleh et al., 2015; Bryce et al,, 2010); Smart et al., 2020). Recent studies have demonstrated that exposure to nanoparticles can induce oxidative DNA damage in TK6 cells, leading to the formation of DNA adducts and the activation of DNA repair pathways (Singh et al., 2009; Gonzalez et al., 2008). This highlights the utility of the TK6 cell line in evaluating the genotoxicity of nanomaterials and other emerging contaminants.

TK6 cells are non-adherent and grow in suspension, displaying similar morphological and functional characteristics to primary human lymphoblasts. TK6 cells are also used to explore the molecular mechanisms underlying the cellular response to oxidative stress, as they are sensitive to reactive oxygen species (ROS) and can activate antioxidant defense mechanisms in response to oxidative damage (Godderis et al., 2012; Li et al., 2020). These properties make the TK6 cell line an important model for studying the effects of environmental and chemical exposures including nanoplastics on human health.

1.5.7. The Raji-B cell line

The Raji-B cell line, derived from the Burkitt's lymphoma of an 11-year-old male, is a widely utilized human B lymphocyte cell line in biomedical research. Established by Pulvertaft (1965), Raji-B cells are characterized by carrying the Epstein-Barr virus (EBV), making them one of the first continuous human cell lines of hematopoietic origin. These cells are frequently used to study B-cell biology, the role of EBV in lymphomagenesis, and immune responses. Raji-B cells express various surface markers typical of B lymphocytes, including CD19, CD20, and CD21, and they have a high capacity for antigen presentation, which makes them a valuable model for studying immune interactions, particularly those involving EBV. Raji-B cells are especially valuable for research into lymphoid malignancies and the mechanisms of viral oncogenesis due to their EBV-

positive status. This characteristic allows researchers to explore the interactions between EBV and the host immune system, including the mechanisms by which EBV transforms B cells and contributes to the development of Burkitt's lymphoma. Additionally, Raji-B cells are utilized in the study of complement-mediated lysis and in assays for detecting immune complexes due to their expression of complement receptors. In genetic and molecular biology studies, Raji-B cells are often employed as a model system for gene editing, transfection, and the study of chromosomal translocations, particularly the c-MYC translocation typical of Burkitt's lymphoma. Their ease of culture, combined with their robust growth characteristics, makes Raji-B cells an ideal choice for various *in vitro* assays, including those assessing cytotoxicity, apoptosis, and cell signaling pathways (Pulvertaft, 1965; Klein et al., 1974; de Thé, 2000). Recent studies have shown that Raji-B cells can be used to investigate the efficacy of novel therapeutic agents, including monoclonal antibodies and small molecule inhibitors targeting B-cell malignancies, by examining their impact on cell viability, proliferation, and apoptosis (Yuan et al., 2024).

Raji-B cells grow in suspension and exhibit morphological and functional properties similar to those of primary B lymphocytes. Their ability to stably maintain EBV episomes, coupled with their well-characterized genetic background, makes Raji-B cells a key model for research in virology, immunology, and oncology. These cells are particularly useful for studying the molecular mechanisms underlying B-cell transformation, viral latency, and immune evasion strategies employed by EBV. Furthermore, they serve as a platform for testing the efficacy and safety of new therapeutic approaches aimed at treating B-cell lymphomas and other EBV-associated diseases.

1.5.8. The MH-S cell line

The MH-S cell line, derived from the alveolar macrophages of a BALB/c mouse, is a widely utilized murine macrophage cell line in immunology and pulmonary research. Established by Myrvik et al. (1977), MH-S cells exhibit characteristics typical of alveolar macrophages, including phagocytic activity and the expression of surface markers such as F4/80, CD11c, and MHC class II molecules. These cells are particularly valuable for studying the immune responses of the lung, including the mechanisms of pathogen recognition, inflammation, and phagocytosis, which are critical in understanding

respiratory diseases. MH-S cells are extensively used in research focused on pulmonary immunity and the response to airborne pathogens, allergens, and environmental toxins. Due to their origin from alveolar macrophages, they serve as a relevant *in vitro* model for examining the role of macrophages in the lungs, especially in the context of infections like influenza and tuberculosis, as well as in chronic lung conditions such asthma and chronic obstructive pulmonary disease (COPD). Their ability to produce cytokines and chemokines in response to various stimuli, such as lipopolysaccharide (LPS) and particulate matter, makes MH-S cells an important tool for studying the inflammatory responses in the lung microenvironment. In addition to their use in immunological studies, MH-S cells are also employed in toxicological research to assess the effects of inhaled substances, including nanoparticles and chemical pollutants, on macrophage function. These cells are highly responsive to oxidative stress and are used to explore the molecular pathways involved in oxidative damage and the activation of antioxidant defenses in alveolar macrophages (Myrvik et al., 1977; Zaynagetdinov et al., 2011; Hussain et al., 2009). Recent studies have demonstrated that MH-S cells can be utilized to investigate the therapeutic potential of anti-inflammatory and antioxidant compounds in mitigating lung inflammation and injury caused by environmental exposures (Park et al., 2015; Karaca et al., 2019).

MH-S cells are adherent and exhibit morphological and functional properties like primary alveolar macrophages, including the ability to engulf and digest pathogens and particles. Their murine origin and ease of culture make MH-S cells a preferred model for studying alveolar macrophage biology, particularly in murine models of lung disease. Additionally, MH-S cells are used to explore the signaling pathways involved in macrophage activation, including the NF-κB and MAPK pathways, which play critical roles in mediating inflammatory responses in the lungs. This cell line is a valuable asset for research in pulmonary immunology, toxicology, and the development of new therapeutic strategies for treating respiratory diseases.

1.6. Research gaps in studying the immunotoxicity of MNPLs

Research into the toxicity of MNPLs reveals several significant gaps, particularly concerning the diversity of polymer types, sizes, and surface characteristics of

nanoplastics. Recent studies highlight that different polymers, such as PS and PE, exhibit distinct behaviors at the cell membrane interface, influenced by their size and surface properties (L. Li et al., 2023). However, there is a lack of comprehensive data on how these variations affect cellular responses, especially in different cell types. For instance, macrophage polarization, a critical immunological property, can be significantly altered upon exposure to nanoplastics, yet the mechanisms remain poorly understood. Studies have shown that NPLs can induce macrophage polarization towards a pro-inflammatory state, but the extent and nature of this response can vary widely depending on the NPL characteristics (Brandts et al., 2023a). Addressing these gaps is crucial for a more accurate assessment of the health risks posed by nanoplastics and for developing standardized methods to study their toxicity (Li et al., 2022b;Atugoda et al., 2023a).

1.6.1. Size effect

Recent studies indicate that the size of NPLs, typically ranging from 1 to 1000 nm, plays a crucial role in their environmental behavior and biological interactions (Atugoda et al., 2023a). Smaller NPLs are more easily transported over long distances and can penetrate cells and tissues more readily than larger particles, leading to potentially more acute toxicological effects (Xie et al., 2023). However, there is a lack of comprehensive data on how different sizes of NPLs affect various cell types and biological systems. For instance, smaller NPLs have been found in human blood, liver, lung cells, and reproductive tissues, but the specific mechanisms of toxicity and the long-term health implications remain poorly understood (Liu et al., 2024). Addressing these gaps is essential for developing standardized methods to assess the health risks posed by NPLs and for creating effective regulatory policies to mitigate their impact.

1.6.2. Type of plastic polymer

Recent studies indicate that various polymers, such as PS, PE, and PP, exhibit distinct behaviors in biological systems due to their unique chemical structures and surface properties (Hossain et al., 2024). However, there is a lack of comprehensive data on how these different polymer types affect cellular responses and toxicity. For instance, PS NPLs have been shown to induce oxidative stress and inflammatory responses in human

lung cells, while PE and PP may have different toxicological profiles (Choe et al., 2021). Additionally, the interaction of these polymers with environmental factors, such as UV radiation and microbial activity, can alter their surface properties and, consequently, their toxicity (Namsheer & Rout, 2021).

1.6.3. Cell type and immunotoxic endpoints

Research into the toxicity of MNPLs reveals several significant gaps, particularly regarding their effects on different cell types and immunological responses. Recent studies have shown that NPLs can induce various inflammatory responses, but the specific mechanisms and consequences remain poorly understood (Summer et al., 2024). For instance, exposure to NPLs has been linked to macrophage polarization, where macrophages shift towards a pro-inflammatory state, potentially leading to chronic inflammation (Jiang et al., 2024). However, the extent of this response can vary widely depending on the type and size of the NPLs, as well as the specific cell type involved (Stock et al., 2022). Additionally, there is limited data on how NPLs interact with other immune cells, such as T cells and dendritic cells, and the long-term immunological consequences of these interactions (Hirt & Body-Malapel, 2020b). Addressing these gaps is crucial for developing a comprehensive understanding of the health risks posed by NPLs and for creating effective regulatory policies to mitigate their impact.

INTRODUCTION

2. Objectives

2. Objectives

Micro- and nano-plastics (MNPLs) are considered emerging pollutants that are widely distributed across all environmental compartments. There is evidence that humans are exposed to MNPLs primarily through inhalation and ingestion, and the small size of these plastic particles may allow for their absorption, systemic distribution, and bioaccumulation. Despite the intensive evaluation of their potential biological effects, their health impacts on humans remain poorly understood. In this context, given the importance of the immune system for human health, the potential immunotoxicity of MNPL exposure is of particular concern.

Therefore, the general objective of this thesis is to generate information that will contribute to a better understanding of the potential impact of MNPL exposure on the immune system. To achieve this general goal, this Thesis aims to cover the following objectives:

1. To study the kinetics and toxicity of polystyrene nanoplastics (PSNPLs, sizes between 50 and 200 nm) in a set of cellular models important for the innate and adaptive immune system.
    1.1. To understand the role of particle size as modulator of the observed kinetic and toxic effects of PSNPLs.
    1.2. To study the differential cell type response to PSNPLs exposure in the different target cells.
2. To study the kinetics and toxicity of true-to-life polyethylene terephthalate nanoplastics (PETNPLs) in alveolar macrophages, focusing on macrophage polarization.
    2.1. To study the relationship between the level of PETNPLs exposure and the observed induced effects

- Objective 1 (and 1.1 and 1.2) will be developed in Chapter 1.
- Objective 2 (and 2.1) will be developed in Chapter 2.

RESULTS

3. Results

3. RESULTS

The results of this thesis are presented in two chapters, each including an article that fulfills the thesis objectives and has been published in peer-reviewed journals.

The two articles, organized according to the thesis objectives, are as follows:

3.1. Chapter 1 (Article 1):

Hazard assessment of different-sized polystyrene nanoplastics in hematopoietic human cell lines.

Chemosphere, 325: 138360 (2023). doi: 10.1016/j.chemosphere.2023.138360

*Impact factor: 8.1. First decile (*Position 32/358 in the Environmental Sciences field)

3.2. Chapter 2 (Article 2):

Harmful effects of true-to-life nanoplastics derived from PET water bottles in human alveolar macrophages.

Environmental Pollution, 348: 123823 (2024). doi: 10.1016/j.envpol.2024.123823.

*Impact factor: 7.6. First quartile (*Position 37/358 in the Environmental Sciences field)

RESULTS

4. Discussion

4. Discussion

The issue of MNPL contamination and accumulation in the environment has garnered considerable public and scientific attention due to the potential health risks and environmental impact associated with these particles. Their unique physical properties make plastics some of the most persistent environmental pollutants, as they resist aging and undergo minimal biological degradation. This durability, while beneficial in many applications, results in long-term pollution challenges and contributes to the accumulation of MNPLs in diverse ecosystems (Rios et al., 2007). The persistent presence of MNPLs across various ecosystems, including marine environments, has led to an increased likelihood of human exposure through consumption. This exposure is of particular concern as, at the nanoscale, plastic particles exhibit distinct physicochemical properties—such as a high surface-to-volume ratio, hydrophobicity, and enhanced diffusion rates—that contribute to their environmental stability and persistence. These properties not only allow MNPLs to endure in ecosystems but also enable them to act as vectors for pathogens and a range of contaminants, including organic pollutants and heavy metals, thereby amplifying potential health risks associated with MNPL ingestion (Ramirez Arenas et al., 2022). Consequently, among the various sizes of plastic particles, there is a critical need for deeper research on nanoplastic particles, as their small size may lead to unique effects not observed with micro- or macro-sized plastic particles. Understanding these nano-specific impacts is essential to assessing their potential risks, especially given that nanoscale plastics may interact differently with biological systems due to their distinct physical and chemical properties (Gangadoo et al., 2020). Thus, concerns about MNPLs extend beyond water and the aquatic food chain, encompassing their impact on plants and soils as well. Evidence shows that MNPLs can exert toxic effects on plants and soil ecosystems, underscoring their role as an emerging pollutant with far-reaching implications for terrestrial environments. This broader perspective highlights the need for research on MNPL contamination across various environmental contexts, as these particles may affect food security and ecosystem health through plant absorption and soil quality degradation (D. Kumar et al., 2024). Despite advances in water treatment and nanoplastic removal technologies, significant challenges remain,

particularly in quantification, analysis, and identification methods. These limitations hinder the ability to conduct precise risk assessments, as current techniques often lack the accuracy needed to effectively measure nanoplastics and assess their potential impacts. Enhanced analytical methods are essential to achieve reliable risk evaluations and to address the complex environmental and health concerns posed by nanoplastics(Keerthana Devi et al., 2022). Recent studies have explored the toxic effects of MNPLs, yet significant gaps remain in understanding how different physicochemical properties, origins, and cellular interactions of nanoplastics contribute to their toxicity. This understanding is essential, as characteristics like chemical composition, size, and shape—especially when mimicking realistic environmental conditions—may substantially influence their toxic effects on human cells. Furthermore, since nanoplastics can cross biological barriers, it is critical to examine their interactions within the human body, particularly in relation to the circulatory system and immune cells, to fully assess their potential health impacts (Blackburn & Green, 2022; Kannan & Vimalkumar, 2021).

Recent studies have underscored the critical role of specific physicochemical properties of nanoplastics (NPLs) in determining their toxicity. These properties—including particle size, surface charge, shape, and chemical composition—significantly influence how NPLs interact with biological systems, potentially enhancing or mitigating their toxic effects. Understanding these interactions is crucial to predicting the health risks associated with NPL exposure and guiding future assessments of their environmental and biological impact (Alqahtani et al., 2023), provide a comprehensive review of the sources, interactions, ecotoxicity, exposure routes, and toxicity mechanisms of microplastics (MPLs) and nanoplastics (NPLs), emphasizing how the small size and large surface area of NPLs enhance their ability to adsorb and transport toxic substances. These properties increase their bioavailability and toxicity, underscoring the importance of understanding these interactions to better predict the health risks associated with NPL exposure and to inform future environmental and biological impact assessments. Similarly, Llorca & Farré (2021b) examined how the size, shape, and surface properties of NPLs influence their toxicological effects, including the induction of oxidative stress and inflammatory lesions, with toxicity varying significantly by cell type. Similarly, Busch et al. (2023) explored

nanoplastics toxicity using advanced stem cell-based in vitro models for intestinal and lung tissues. Their findings suggest that stem cell-based models more accurately reflect human physiology compared to traditional cancer cell line models, underscoring the importance of selecting relevant cell types in toxicity studies. These insights collectively highlight how particle properties and biological model selection critically shape our understanding of NPL toxicity across different physiological contexts. Additionally, Atugoda et al. (2023b) reviewed the biological effects of NPLs on different taxonomic groups, noting that smaller particles facilitate penetration across cell membranes, leading to increased cytotoxicity. NPLs have been shown to induce significant immune responses. Thus, a study by Alijagic et al. (2023b) demonstrated that exposure to NPLs can activate the NLRP3 inflammasome, leading to the production of cytokines and the activation of immune cells, such as T cells, which are crucial for initiating immune response. Also, Alijagic & Särndahl (2024) indicated that NPLs can interact with epithelial barriers, potentially triggering a cascade of signaling events that disrupt oxidative and inflammatory balance, causing immune cell toxicity.

Therefore, as detailed in two separate studies within the results section of this Thesis, our objective was to investigate the adverse health effects of NPLs with a particular focus on size, origin, and cell type. To achieve this, we utilized various sizes of polystyrene nanoplastics. Additionally, to replicate real-world exposure conditions, we produced and employed laboratory-produced true-to-life PET NPLs obtained from the degradation of real plastic bottles. These were tested on different human immune cell types to address the gaps identified in the previous sections.

4.1. Hazardous effects of PSNPLs with different sizes on human immune cell lines

One of the crucial factors in studying the adverse impact of emergent pollutants, including NPLs, is the physiochemical properties of those particles because of the direct impact on their toxicity by various outcomes when interacting with human cells. Among all features, the size and the type of polymer in studying NPLs toxicity is an important research gap nowadays to know in deep about the impact of the NPLs and to be able to compare the effects based on their physical and chemical properties. For instance, recent studies

indicate that smaller particles often show increased toxicity due to their larger surface area and enhanced ability to penetrate biological membranes (Li & Li, 2024). This improved penetration can result in greater cellular uptake and interaction with intracellular components, leading to more significant toxic effects. For example, research has shown that smaller NPLs can cause oxidative stress, inflammation, and even genotoxicity in various cell types (Thornton Hampton et al., 2022) These effects are linked to the particles' capacity to produce reactive oxygen species (ROS) and disrupt cellular balance. Furthermore, smaller NPLs have been observed to cross biological barriers, such as the blood-brain barrier, which may result in potential neurotoxic effects (Płuciennik et al., 2024) Additionally, the environmental behavior of NPLs is affected by their size. Smaller particles are more likely to cluster together, and form aggregates and this fact affects their toxicity (Prüst et al., 2020) This can have significant consequences for ecosystem health and human exposure to NPLs.

On the other side, the study models and their characteristics are also important since each model can respond differently to various contaminants. This fact is true for the different cell lines as well since different cell types can greatly affect how toxicological data is interpreted. For example, PSNPLs have been found to cause cytotoxic and genotoxic effects in various cell lines, with the severity of these effects varying according to the specific biological processes of each cell type (Manikanthaand Tulasi, 2023). This variability highlights the importance of choosing suitable cell models that accurately represent the target tissues or organisms being studied. Additionally, the size, charge, and concentration of NPLs in combination with different cell types play a crucial role in their toxic effects, with smaller particles typically showing greater cytotoxicity due to their enhanced ability to penetrate cellular membranes and trigger oxidative stress (Płuciennik et al., 2024). Research has shown that NPLs can lead to oxidative damage in proteins, lipids, and DNA, resulting in cell death through processes like apoptosis and necrosis (Manikanthaand Tulasi, 2023). These results underscore the necessity of considering cell type-specific responses when evaluating the potential health risks associated with NPLs. These systemic effects are often driven by the interaction of NPLs with particular cell types in various organs, further stressing the need for a thorough understanding of cell type-specific toxicological responses.

DISCUSSION

Based on the mentioned information, in the first study (Chapter 1) we delved to find a robust and concise answer to the question of how the particle size and cell type variabilities can affect the toxicity potential of NPLs by using pristine PSNPLs with different sizes of 50 nm, 200 nm, and 500 nm and selecting human hematopoietic cell lines, Raji-B, THP-1, and TK6. The mentioned sizes were selected to represent a variety of nanoplastic dimensions that are frequently found in environmental and biological settings. Smaller particles, like those measuring 50 nm, are recognized for their high surface area-to-volume ratio, which can enhance reactivity and the potential for cellular uptake. This range of sizes enables us to test the hypothesis that smaller nanoplastics might be more toxic due to their greater ability to penetrate cellular membranes and trigger oxidative stress. On the other hand, larger particles, such as those at 500 nm, may interact with cells differently, possibly resulting in varied toxicological effects. By incorporating an intermediate size of 200 nm, we can investigate the size-dependent impacts of nanoplastics in a more detailed way, offering a thorough understanding of how particle size affects toxicity. By utilizing these three cell lines, we can capture a wide range of hematopoietic cell responses, encompassing both adaptive and innate immunity as well as genotoxicity. This strategy enables us to evaluate the varying toxicological effects of nanoplastics across different cell types, thus strengthening the reliability and relevance of our results. The integration of various nanoplastic sizes and distinct hematopoietic cell lines in our research offers a thorough framework for comprehending the intricate interactions between NPLs and biological systems. This approach allows us to clarify the size-dependent and cell-type-specific toxic effects of NPLs, providing important insights into the field of nanotoxicology.

4.1.1. Particle size and cell type affect the interaction between PSNPLs and hematopoietic cells

Based on the outputs of our study which was explained in Chapter 1, we hypothesize that particle size can affect the toxicity and interaction of PSNPLs with human cells. After analyzing the data obtained, we concluded that different-sized PSNPLs show various effects that are driven by other modes of interaction for each size. For instance, the internalization ability of the PSNPLs has an indirect relation with their size which means,

the smaller sizes like PSNPLs 50 nm, 200 nm, and 500 nm show high to low internalization rates, respectively, in THP-1 and Raji-B cells. Similarly, the sizes dependent internalization of PSNPLs assessed in basophilic leukemia (RBL-2H3) cells, and the data showed that the 50 nm PSNPLs internalized more than PSNPLs 50 nm after 6 h of exposure and, in addition, the smaller size PSNPLs reached a plateau step ten times sooner than PSNPLs 500 nm (Liu et al., 2021). This pattern was observed in HeLa cells that based on the correlation between the PSNPLs size and the internalization percentage, the smaller the PSNPLs are the higher the rate of internalization occurs. This could be a result of different mechanisms of internalization based on the particle size since the smaller NPLs can enter the cells via passive diffusion but the larger sizes of NPLs have an active transport mechanism of entry to the cells that relies on caveolae and clathrin-mediated endocytosis (Ruan et al., 2023). Also, in another research (Zhang et al., 2022) authors indicated that the uptake of PSNPLs by Caco-2 and A549 cell lines is influenced by the size of the particles. The smaller particles (PS-70) were absorbed more effectively than the larger ones (PS-200 and PS-500), as demonstrated by confocal microscopy and flow cytometry. This implies that smaller particles are more accessible to these cells. In a similar way, Banerjee et al. (2022) found that 50 nm PSNPLs were significantly taken up by HepG2 cells compared to the 200 nm and 500 nm particles, as observed through confocal microscopy. Moreover, it seems that the smallest particles (PS-70 nm) can penetrate cells through various pathways, including clathrin- and caveolae-mediated endocytosis, as well as phagocytosis. On the other hand, the larger particles (PS-200 and PS-500 nm) can only enter cells via phagocytosis. This variation in entry mechanisms likely accounts for the more efficient internalization of the smallest nanoparticles. Despite size-dependent internalization in THP-1 and Raji-B cells, TK6 uptake data by flow cytometry does not show this tendency which indicates the effect of cell type on PSNPLs toxicity as well as particle size. The matter of cell type in connection with particle size is another interdisciplinary factor that may affect the cellular uptake of NPLs in the study of Choi et al. (2024) they confirmed that the size-dependent internalization of PSNPLs belongs to the cell types since the internalization of 50–100 nm PSNPLs depends on enterocytes and over 500 nm PSNPLs depends on M cells when they assessed the three sizes (50, 100, 500 nm) of PSNPLs in mono-, co-, and tri-cultures

of Caco-2 cells, HT29-MTX-E12 cells, and M cells. In our study, the usage of different analytical tools for cellular internalization in the present study was justified as no one tool could completely capture the NPL's internalization thoroughly due to various factors. For instance, the low atomic number of polymers in MNPLs could cause them to scatter electrons weakly resulting in poor contrast under TEM (Sawyer et al., 2008). In addition, it warrants laborious sample preparation and time consumption which possibly makes it irrelevant to analyze the high number of particles needed for representative studies (Ivleva, 2021). Moreover, MNPLs labeled with fluorophores could possibly shed the fluorophores in a cell culture medium thereby the observation of fluorescence intracellularly might be due to free fluorophores (Tenuta et al., 2011). Also, the potential leaching effect of fluorophores from the labeled PSNPLs was reported (Catarino et al., 2019). Fluorescent-labeled PSNPLs (500–1000 nm) were not able to cross the epithelial barrier of zebrafish larvae rather fluorophores alone were detected in the internal tissues. Apart, the internalization of PSNPLs could be read in flow cytometry due to an increase in the cell complexity; however, the tendency of PSNPLs to adhere to the cell surface may also reflect the increase in cell volume thereby affecting the actual estimation of their internalization in that technique (Lesniak et al., 2013); (Zhang et al., 2022).

4.1.2. Toxic response to PSNPLs is associated with the particle size and cell type influence

Since the PSNPL's internalization capacity varies based on their size and cell response, their size-dependent adverse biological effects are inevitable. This hypothesis was proved with our study in chapter one and the focus was on the cell viability, reactive oxygen species induction, and mitochondrial membrane potential decrease as the key markers of cellular toxic and damage response. Going through the results, we can observe the size and cell type effect meaning that the ROS induction differs among Raji-B, THP-1, and TK6. As previously mentioned in various studies, the smaller size brings more adverse effects, but this scenario is not always general due to the different mechanisms of interactions between particles and cells. For instance, in our study, PSNPLs 200 nm showed the most effect in ROS generation in all cell lines, while PSNPLs 500 nm, and PSNPLs 50 nm showed moderate to low ROS induction respectively. In addition, the cells

showed their different tendency in response to PSNPLs different sizes mentioning that PSNPLs 500 nm only induced ROS in the Raji-B cell line while in other cells, PSNPLs 200 nm were the most impactful particle size. The generation of reactive oxygen species (ROS) is a well-known cellular marker in response to nanoparticles, and the degree of this response can vary greatly based on the size of the nanoparticles. For instance, larger nanoparticles have a greater surface area, which could result in increased reactivity and ROS generation, possibly explaining why 50 nm PSNPLs do not induce ROS in any of the cell lines (Dayem et al., 2017). Another associated factor for this result could be the cellular membrane damage that PSNPLs 500 nm cause in comparison to smaller PSNPLs like 100 nm that are internalized to the cell and aggregated in the cytoplasm (Lu et al., 2022). The ROS response also varies with cell type and the specific endocytosis pathways that different nanoparticle sizes engage. For example, smaller nanoparticles can penetrate cells more easily and stimulate intracellular ROS production through mechanisms like mitochondrial dysfunction and the activation of NADPH oxidase, which are more evident across a variety of cell types (Manke et al., 2013). On the other hand, larger nanoparticles may have a more pronounced effect on certain cell types, such as Raji-B cells, due to their enhanced uptake through macropinocytosis, resulting in localized ROS generation without necessarily impacting other cells (Kettler et al., 2014). NPLs may heighten the intracellular level of ROS through various pathways, such as mitochondrial dysfunction, leakage of mitochondrial DNA, and activation of the cGAS-STING pathway, leading to inflammation and production of ROS (Wang et al., 2024). It can induce lysosomal damage after endocytosis and the release of enzymes increases the ROS level (Hua & Wang, 2022). Besides, NPLs can induce oxidative stress through the generation of ROS or even through interference with cellular antioxidant defenses. Also, the functionalization of NPLs with several chemical groups increases the interaction with cellular components, which, in turn, enhances the amount of ROS (Płuciennik et al., 2024).

In addition to ROS induction, in this study, we observed the different mitochondrial membrane potential responses toward PSNPLs. Regardless of particle size, cell type is associated with the effects of PSNPLs as well. The differences in results of mitochondrial membrane potential (MMP) assays across various cell lines when exposed to PSNPLs

highlight the importance of cell type variability along with particle size in toxicological research. For instance, the TK6 cell line showed no change in MMP, while significant decreases were noted in THP-1 and Raji-B cells. This variation can be linked to several factors specific to the cell lines like the least internalization rate of PSNPLs in TK6 compared to other cell types in this study and the lack of evidence about the interaction of PSNPLs with the mitochondria in TEM micrographs. Although no specific study compares the size effect of PSNPLs on mitochondrial dysfunction in THP-1 cells, our data indicate that Raji-B and THP-1 cells are more responsive to different sizes of PSNPLs compared to TK6 cells. Our data on the size-dependent loss of mitochondrial membrane potential (MMP) in Raji-B cells align with findings from a study on Caco-2 cells exposed to PSMPLs of 0.1 and 5 μm sizes. The study showed that cells treated with 5 μm PSMPLs experienced a greater loss of MMP compared to those treated with 0.1 μm PSMPLs (Wu et al., 2019b). Furthermore, amino-modified PSNPLs (50 nm) had a notable impact on mitochondrial metabolic activity and MMP loss in a time- and dose-dependent manner in alveolar macrophages. Similarly, amino-modified PS-20, unmodified PS-20, and PS-50 nm induced MMP loss, increased proton leak from mitochondria, and elevated ATP generation and mitochondrial oxidative phosphorylation in a dose-dependent manner in A549 cells (Shi et al., 2022). In addition, Huang et al., 2023 showed the size-dependent effect of PSNPLs in mitochondrial disruption mentioning that the diminished MMP in AML-12 cells is associated with the particle size because, between tested particle sizes of 100 nm and 20nm, the PSNPLs 20 nm showed greater fluorescent signal representing the more mitochondrial membrane potential decrease upon localization in mitochondria (Huang et al., 2023).

Despite all of the effects caused by PSNPLs of different sizes on human hematopoietic cell lines, no significant alterations in cell viability were observed in this study that aligns with previous studies (Cortés et al., 2020; Rubio et al., 2020b), when they assessed the effects of different-sized PSNPLs on Caco-2 and human hematopoietic cell lines. This alignment not only confirms our result regarding the mild cytotoxic effects of PSNPLs but also provides valuable evidence that the lack of dramatic change in cell viability does not necessarily comply with the deep cellular mechanisms that happen in response to MNPLs.

4.2. Harmful effects of true-to-life nanoplastics derived from PET water bottles in human alveolar macrophages

Studying the adverse effects of NPLs undoubtedly should consider the real factors associated with the environmentally derived NPLs that result from their natural degradation and translocation to simulate the realistic human exposure factors which are crucial in toxicological studies. To do so, in Chapter 2 of this Thesis, we provide a study about the true-to-life NPLs derived from PET water bottles in human alveolar macrophages which shows at the same time the necessity of using true-to-life NPLs and considering the PET NPLs as type of plastic polymer, and finally used the alveolar macrophage as a study model to investigate the immune response toward NPLs. Human exposure to MNPLs can happen through various sources of commercial plastics, entering the body through oral, inhalation, and skin contact (Llorca & Farré, 2021c). Breathing in atmospheric MNPLs may cause oxidative stress and inflammation in lung tissue, as these particles can reach the lungs. In this context, immune responses involving inflammatory cells, especially human alveolar macrophages, become significant. These macrophages can engulf MNPLs through a process called phagocytosis, which is vital for the body's immune defense. However, long-term exposure to MNPLs may hinder macrophage function (Bruno et al., 2024; Rodrigues et al., 2022). Therefore, it is crucial to explore the health effects of MNPLs, particularly focusing on immune responses at both cellular and tissue levels.

4.2.1. The importance of using true-to-life nanoplastic

Lab-made, realistic NPLs are crucial for investigating their toxic effects on human cells because environmental NPLs vary greatly in size, shape, and chemical properties due to degradation. This variability makes toxicological evaluations challenging, hindering the identification of specific cellular and molecular impacts. By producing and characterizing controlled NPLs in the lab, we are able to standardize important particle features which are vital for consistent and accurate research. This approach helps to clarify how variations in size, shape, or chemical modifications affect toxic responses, such as cellular uptake, oxidative stress, and inflammation. True-to-life NPLs provide a clear

understanding of the potential health risks to humans from environmental exposure (Enyoh et al., 2019; Gigault et al., 2018). Previous studies on NPLs mostly focused on commercially available NPLs with perfect spherical shape and size. Despite their engineered and standardized features to represent the size of the environmental NPLs, they cannot accurately reflect the irregular surface characteristics and shape that result from natural degradation. This difference can bring several questions and doubts about the real simulation of NPLs exposure (Yong & Du, 2023). From a more detailed point of view, by considering their sources we know that they have two main origins, one is derived from plastic manufactured materials like food packaging, textile fibers, plastic resins, and 3D printing which are defined as primary NPLs and the second origin is formed when larger plastic materials, such as plastic bags, bottles, and fishing gear, degrade over time due to environmental factors like weathering, and biological activities from animals and microbes. When we assess the NPLs toxic effect using perfectly spherical manufactured v we cannot represent the real changes that occurred on the plastic materials due to the environmental degradation (da Costa, 2018). In 2021, Kefer et al. established a comparative overview regarding the differences between engineered and environmental MPLs and the results showed that surface characteristics and morphology are heterogeneous among the environmentally derived MPLs and the most abundant form of the particles are fibers showing the lack of real simulation in assessing the environmental MNPLs since this various morphology and characteristics affect both the degradation to NPLs and their effect on human body considering the fact that the model of plastic production has two approaches including bottom-up and top-down methods and while in the industry the first method is utilizing that produces particles from basal molecular parts in a uniform patter, is not going to represent the diverse fractions of plastics derived from environmental degradation since the real condition that happens in the environment usually comply with the top-down method which generates particles by breaking down the larger objects and produce particles with various shapes and morphologies (Kefer et al., 2021).

Despite its crucial effect, few studies currently use true-to-life NPLs to study their toxic effects. To cover this gap, we aimed to produce, optimize, and characterize the PETNPLs in the laboratory, as mentioned in Chapter 2.

4.2.2. PETNPLs affect immune system through inhalation

Polyethylene terephthalate nanoplastics abbreviated to PETNPLs is a polymer thermoplastic material derived from petroleum that has become the most widely used plastic globally due to its useful characteristics like durability, high transparency, and lightweight and its complex structure makes PET difficult to degrade, allowing it to persist in nature for extended periods (Jin et al., 2023). Regardless of its important role as an environmental contaminant, this polymer has been studied less than other counterparts as other NPLspolymers are available (Villacorta et al., 2022). There are few studies accounting for this polymer type since 2016 that assessed its toxic effect, initiated by some *in vivo* studies like Jemec et al. (2016) that used PET microfibers from textiles and assessed their toxic effects in *Daphnia magna*. Some *in vitro* studies have been done like Magrì et al. (2021b) which studied the interaction of PETNPLs with water contaminants and their effect on Caco-2 cells. On the other side, Gettings et al. (2024) showed the exposure impacts of PETNPLs on human brain vascular pericytes. Considering their ability to enter the human body through different routes, including respiratory organs which are the primary target site for the toxic effects of airborne nanoplastics, currently, few data exist on the toxic effect of PETNPLs on this organ (Lim et al., 2021).In 2022, Zhang et al. (2022) assessed the toxic impacts of PETNPLs on A549 cells representing the pulmonary exposure that showed increased ROS and diminished mitochondrial membrane potential. Another study investigated the effects of true-to-life PETNPLs on human nasal epithelial cells. Despite the increased concern about their presence in the atmosphere and their potential ability to enter the human body, still there is negligible evidence regarding PETNPLs toxicity on the respiratory system (Annangi, Villacorta, Vela, et al., 2023). In addition to the direct impact of PETNPLs on the human pulmonary system, there is a missing part that should be noted: the interaction of the immune system in the route of PETNPLs to the respiratory organs and pulmonary system. There is evidence showing that human inhalation of atmospheric NPLs can result in excessive ROS induction and inflammation in lung tissue. This occurs because particles can reach the lungs, where they interact with immune cells, particularly alveolar macrophages. These immune cells, which are a crucial factor for the human body’s immune defense

system can internalize particles through phagocytosis; however, prolonged exposure to nanoplastics may affect the functional aspects of these macrophages (Allard et al., 2018; Q. Chen et al., 2022; Jenner et al., 2022). To cover this research gap and to have a better understanding of the interactions between PETNPLs and alveolar macrophages which is essential for developing strategies to mitigate their harmful effects we have explored the toxic and immunotoxic effects of PETNPLs on alveolar macrophages, as presented in Chapter 2 of this Thesis.

In this study, we aimed to assess the presence of PETNPLs in alveolar macrophages (MH-S), their toxic effects on this cell type, and the immunotoxic response toward these NPLs. The utilized approach for this aim was initiated by isolating and sorting the cells that internalized PETNPLs to further study this specific population to obtain specific responses from the exposed cells and exclude them from the heterogeneous population. This cell sorting approach has been classically used to select and isolate a desired population of cells from a heterogeneous culture (Ciaglia et al., 2019; Latroche et al., 2018). The isolated populations tested in this study showed that PETNPLs can induce ROS and affect mitochondrial membrane potential, depending on time and concentration of exposure. Similar studies confirm our findings about the effects of NPLs in general and PETNPLs in particular on alveolar macrophages. Tapak et al.(2023a) research highlighted the critical role of alveolar macrophages in responding to particulate matter

exposure, including NPLs. Their findings emphasized that NPLs exposure can trigger ROS, resulting in acute damage and inflammation in these cells which is consistent with our findings. Additionally,Allard et al. (2018) underscore that prolonged exposure to nanoplastics can impair macrophage function, weakening the pulmonary immune defense. Reactive oxygen species are highly reactive molecules that result in oxidative stress, leading to damage cellular components including lipids, proteins, and DNA in alveolar macrophages that can impair cell function (Herold, 2023). One of the primary roles of ROS in alveolar macrophages is to mediate antimicrobial responses. ROS are produced during the reaction named oxidative burst, a rapid release of reactive oxygen species that cells utilize to kill pathogens. However, excessive ROS can also lead to chronic inflammation via activating signaling pathways that promote the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Herold, 2023). These cytokines further activate other immune cells, amplifying the inflammatory response. Also, exposure of alveolar macrophages to environmental particulate matters induces the production of ROS within the airways of the lung (Fessler & Summer, 2016)and this increase in ROS leads to an increase in macrophage phagocytosis, but the overproduction of ROS can induce inflammation (Fan & Fan, 2018; Korns et al., 2011).

Another effect obtained from PETNPLs exposure to alveolar macrophages in our study was the decrease in mitochondrial membrane potential in a time and concentration-dependent manner. This reduced MMP indicates one of the features that, in association with other factors, can lead to mitochondrial dysfunction (Belchamber et al., 2019). This mitochondrial dysfunction in alveolar macrophages can impair their ability to produce energy, leading to reduced phagocytic activity and decreased ability to clear pathogens (Lugg et al., 2022; Tapak et al., 2023b). Similar effects derived from nanoparticles were observed in the study of Soliman et al. (2020) when they showed that carbon nanotubes can induce mitochondrial impairment in alveolar macrophages. Pulskamp et al. (2007) observed decreased MMP and increased ROS in alveolar macrophages in response to carbon nanotubes. Another study focused on the particulate matter on rat alveolar macrophages, proved mitochondrial membrane impairment due to the carbon black nanoparticles in combination with heavy metals (Guan et al., 2020). Mitochondrial membrane hyperpolarization and increased ROS were observed in response to amino-

modified PSNPLsin alveolar macrophages in the study by Deville et al. (2020). Therefore, the mitochondrial function in alveolar macrophages is affected by different nanoparticles but no studies about the PETNPL's effects on this organelle were studied in this type of immune cells. The MMP and ROS are important factors in immune cell types due to their role promoting inflammation. ROS can activate the NF-kB pathway which is a key regulator of inflammation that can lead to transcription of pro-inflammatory genes (Fan & Fan, 2018). In addition, mitochondrial dysfunction can release mitochondrial DNA (mtDNA) into the cytosol that acts as a damage-associated molecular pattern and further simulates the inflammatory pathways (Wei et al., 2024). In addition, mtDNA can activate the NLRP3 inflammasome, and promote the release of interleukin-1β and interleukin-18 (De Gaetano et al., 2021).

Considering all mentioned studies and the potential relation between reactive oxygen species induction, mitochondrial damage axis, and inflammation response in immune cells especially alveolar macrophages we can conclude the immunotoxic effects of PETNPLs on this cell line, as outlined in our study in Chapter 2, even though this is not covering all the aspects of the immune response toward this NPLs type. In this study, we also delved into answering to the question that exposure to PETNPLs may alter the macrophage phenotype from M0 stage to M1 (as a pro-inflammatory phenotype) and M2 (as an anti-inflammatory phenotype) which is a crucial factor that affects the immune system and function.

4.2.3. PETNPLs exposure leads to macrophage polarization in MH-S cells

Macrophages are highly adapting cells due to their plasticity and can polarize into either pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes in response to environmental signals, playing essential roles in immune responses, tissue repair, and homeostasis. The balance between M1 and M2 phenotypes in macrophages is crucial to maintaining immune balance and preventing chronic inflammatory diseases (Deng et al., 2023). For instance, the exaggeration of polarization imbalance in macrophages has an essential role in the pathogenesis of autoimmune and autoinflammatory diseases (Peng et al., 2023; Tardito et al., 2019). Contemplating the importance of macrophage phenotypic balance, and the potential interfering role of NPLs on this balance, we delved

to assess the effects of PETNPLs on macrophage polarization in MH-S cells that this study well positioned in Chapter 2 of this Thesis. The role of NPLs in macrophage polarization comes from previous studies mentioning their ability to affect this transition via different signaling pathways such as the ROS-dependent pathways (Fan et al., 2022; Jiang et al., 2024). Such studies are focused on macrophage polarization but the used NPLs in these works were commercially available PSNPLs, and despite their positive action on macrophage polarization, exposure to nanoplastics mimicking the real environmental feature remains unknown, especially for polymer types like PET which consisted of the most abundant type of NPLs (Villacorta et al., 2022). We assessed macrophage polarization as a result of PETNPLs exposure in the sorted population of cells that contained PETNPLs and, as a consequence, our data showed that PETNPLs can result in M0 to M1, and M0 to M2 polarization in MH-S cells in a time and concentration-dependent manner.

This work captured the alterations in immune responses, using the expression of toll-like receptors on the surface of mouse alveolar macrophages-MH-S cells as representatives of the transition of M0 subtypes to M1 and M2. The results showed that the PETNPLs remarkably affected the polarization of MH-S cells from M0 to M1 and M2 subtypes. Importantly, there is a clear trend in the way the treated cells with PETNPLs are drifting significantly towards M1 subtype macrophages, as seen with increased expression of the biomarker CD86, which decreased at higher concentrations when compared to untreated cells at both time points suggesting that PETNPLs do induce a pro-inflammatory response herein. These results agree with the pro-inflammatory polarization observed in MH-S-exposed PSNPLs, evidenced by the biomarker CD38 (Collin-Faure et al., 2022). Besides, consistent M0 to M1 polarization occurred after 3–24 h in sorted cells that have internalized PETNPLs. The pro-inflammatory M1 polarization effect of PETNPLs was therefore temporally stable in these cells. Meanwhile, no similar studies in literature confirm the relevance of exposure time, though its importance to macrophage polarization has been noted in the study of Murray (2017). Other indirect studies on murine macrophages RAW264.7 exposed to PSMNPLs, and human lung macrophage cells, respectively (Wang et al., 2023;Wang et al., 2023), suggested its induction in macrophage polarization. Other research was done on the action of various

sizes of NPLs, showing different cytokine secretion profiles, shifting to pro-inflammatory M1 and not M2 phenotypes (Wolff et al., 2023b). Our findings were not limited to M1 polarization, and the M2 polarization detected in the gated cells containing PETNPLs demonstrating polarization to the M2 subtype, as confirmed by higher expression of the CD163 surface biomarker versus untreated cells. These effects were more pronounced after 24 h of treatment at all tested concentrations, indicating a subpopulation of M2 macrophages involved in anti-inflammatory or wound-healing responses along with the M1 subtype. Thus, the distinct M2 responses between 3 and 24 h of exposure may reflect time-dependent macrophage immune responses. At the beginning stage, macrophages perform a pro-inflammatory response toward insults but have been shown to switch toward an M2 phenotype at the late stage to nullify the pro-inflammatory outcomes (Murray, 2017). At the same time, there was M2 polarization in the rainbow trout macrophages (RT-HKM) when exposed to PSNPLs, typical of an anti-inflammatory response (Brandts et al., 2023b); similarly, it occurred in human monocyte-derived macrophages (Wolff et al., 2023b). More recently, in an *in vivo* mouse model, using real-life PETMPLs and RNA-seq analysis, it has been shown that exposure impairs immune homeostasis (Harusato et al., 2023). These findings, along with our results, suggest the potential treatment of nanoplastics exposure on human health mentioning the immune response alterations.

CONCLUSIONS

5. Conclusions

5. Conclusions

In accordance with the objectives raised in the frame of this Thesis and the discussion of the obtained results, we conclude that:

1. PSNPLs (50-500 nm) are effectively internalized by immune cells, leading to ROS induction and impairment of mitochondrial membrane potential.
2. These observed PSNPLs-induced effects are modulated by particle size, as a size-dependent response is observed under identical exposure conditions.
3. Additionally, the cellular model used as the target influences the effects induced by PSNPLs, as a differential response is observed in THP-1, TK6 or Raji-B under the same exposure conditions.
4. True-to-life PETNPLs are internalized in alveolar macrophages following a heterogeneous pattern where only a subset of cells internalizes a significant number of particles.
5. In this subset of exposed cells, PETNPLs impair the immune response by disrupting the balance between M0, M1, and M2 macrophage subtypes.
6. The use of cell sorting to isolate cells that have effectively internalized plastic particles is highly useful for eliminating the bias introduced by cells that do not internalize the particles. We recommend this approach for studying the effects of NPL exposure in vitro, particularly when the cell line of interest shows low internalization capacity.

CONCLUSIONS

6. References

6.References

Acharjee, S. A., Bharali, P., Gogoi, B., Sorhie, V., Walling, B., & Alemtoshi. (2023). PHA-Based Bioplastic: a Potential Alternative to Address Microplastic Pollution. In *Water, Air, and Soil Pollution* (Vol. 234, Issue 1). Institute for Ionics. https://doi.org/10.1007/s11270-022-06029-2

Alabi, O., Ologbonjaye, K., Awosolu, O., & Alalade, O. (2019). Public and Environmental Health Effects of Plastic Wastes Disposal: A Review. *Journal of Toxicology and Risk Assessment*, *5*. https://doi.org/10.23937/2572-4061.1510021

Alaraby, M., Abass, D., Domenech, J., Hernández, A., & Marcos, R. (2022a). Hazard assessment of ingested polystyrene nanoplastics in Drosophila larvae. *Environ. Sci.: Nano*, *9*(5), 1845–1857. https://doi.org/10.1039/D1EN01199E

Alaraby, M., Abass, D., Domenech, J., Hernández, A., & Marcos, R. (2022b). Hazard assessment of ingested polystyrene nanoplastics in Drosophila larvae. *Environ. Sci.: Nano*, *9*(5), 1845–1857. https://doi.org/10.1039/D1EN01199E

Alaraby, M., Villacorta, A., Abass, D., Hernández, A., & Marcos, R. (2023). The hazardous impact of true-to-life PET nanoplastics in Drosophila. *Science of the Total Environment*, *863*. https://doi.org/10.1016/j.scitotenv.2022.160954

Ali, N., Katsouli, J., Marczylo, E. L., Gant, T. W., Wright, S., & Bernardino de la Serna, J. (2024a). The potential impacts of micro-and-nano plastics on various organ systems in humans. In *eBioMedicine* (Vol. 99). Elsevier B.V. https://doi.org/10.1016/j.ebiom.2023.104901

Ali, N., Katsouli, J., Marczylo, E. L., Gant, T. W., Wright, S., & Bernardino de la Serna, J. (2024b). The potential impacts of micro-and-nano plastics on various organ systems in humans. *EBioMedicine*, *99*, 104901. https://doi.org/10.1016/j.ebiom.2023.104901

Alijagic, A., Hedbrant, A., Persson, A., Larsson, M., Engwall, M., & Särndahl, E. (2023a). NLRP3 inflammasome as a sensor of micro- and nanoplastics immunotoxicity. In *Frontiers in Immunology* (Vol. 14). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2023.1178434

Alijagic, A., Hedbrant, A., Persson, A., Larsson, M., Engwall, M., & Särndahl, E. (2023b). NLRP3 inflammasome as a sensor of micro- and nanoplastics immunotoxicity. In *Frontiers in Immunology* (Vol. 14). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2023.1178434

Alijagic, A., & Särndahl, E. (2024). Can evolutionary immunology decode micro and nanoplastic challenges? *Frontiers in Immunology*, *15*. https://doi.org/10.3389/fimmu.2024.1404360

Allard, B., Panariti, A., & Martin, J. G. (2018). Alveolar Macrophages in the Resolution of Inflammation, Tissue Repair, and Tolerance to Infection. *Frontiers in Immunology*, *9*. https://doi.org/10.3389/fimmu.2018.01777

Allen, S., Allen, D., Phoenix, V. R., Le Roux, G., Durántez Jiménez, P., Simonneau, A., Binet, S., & Galop, D. (2019). Atmospheric transport and deposition of microplastics in a remote mountain catchment. *Nature Geoscience*, *12*(5), 339–344. https://doi.org/10.1038/s41561-019-0335-5

REFERENCES

Alqahtani, S., Alqahtani, S., Saquib, Q., & Mohiddin, F. (2023). Toxicological impact of microplastics and nanoplastics on humans: understanding the mechanistic aspect of the interaction. In *Frontiers in Toxicology* (Vol. 5). Frontiers Media SA. https://doi.org/10.3389/ftox.2023.1193386

Alzaben, M., Burve, R., Loeschner, K., Møller, P., & Roursgaard, M. (2023). Nanoplastics from ground polyethylene terephthalate food containers: Genotoxicity in human lung epithelial A549 cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *892*, 503705. https://doi.org/https://doi.org/10.1016/j.mrgentox.2023.503705

Andrady, A. L. (2017). The plastic in microplastics: A review. *Marine Pollution Bulletin*, *119*(1), 12–22. https://doi.org/https://doi.org/10.1016/j.marpolbul.2017.01.082

Andrady, A. L., & Neal, M. A. (2009). Applications and societal benefits of plastics. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1526), 1977–1984. https://doi.org/10.1098/rstb.2008.0304

Annangi, B., Villacorta, A., López-Mesas, M., Fuentes-Cebrian, V., Marcos, R., & Hernández, A. (2023). Hazard Assessment of Polystyrene Nanoplastics in Primary Human Nasal Epithelial Cells, Focusing on the Autophagic Effects. *Biomolecules*, *13*(2). https://doi.org/10.3390/biom13020220

Annangi, B., Villacorta, A., Vela, L., Tavakolpournegari, A., Marcos, R., & Hernández, A. (2023). Effects of true-to-life PET nanoplastics using primary human nasal epithelial cells. *Environmental Toxicology and Pharmacology*, *100*. https://doi.org/10.1016/j.etap.2023.104140

Arias-Andres Maria and Rojas-Jimenez, K. (2022). Ecological and Public Health Effects of Microplastics Pollution. In M. Z. Hashmi (Ed.), *Microplastic Pollution: Environmental Occurrence and Treatment Technologies* (pp. 429–444). Springer International Publishing. https://doi.org/10.1007/978-3-030-89220-3_19

Arribas Arranz, J., Villacorta, A., Rubio, L., García-Rodríguez, A., Sánchez, G., Llorca, M., Farre, M., Ferrer, J. F., Marcos, R., & Hernández, A. (2024). Kinetics and toxicity of nanoplastics in ex vivo exposed human whole blood as a model to understand their impact on human health. *Science of the Total Environment*, *948*. https://doi.org/10.1016/j.scitotenv.2024.174725

Atugoda, T., Piyumali, H., Wijesekara, H., Sonne, C., Lam, S. S., Mahatantila, K., & Vithanage, M. (2023a). Nanoplastic occurrence, transformation and toxicity: a review. In *Environmental Chemistry Letters* (Vol. 21, Issue 1, pp. 363–381). Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s10311-022-01479-w

Atugoda, T., Piyumali, H., Wijesekara, H., Sonne, C., Lam, S. S., Mahatantila, K., & Vithanage, M. (2023b). Nanoplastic occurrence, transformation and toxicity: a review. In *Environmental Chemistry Letters* (Vol. 21, Issue 1, pp. 363–381). Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s10311-022-01479-w

Auffan, M., Rose, J., Bottero, J. Y., Lowry, G. V., Jolivet, J. P., & Wiesner, M. R. (2009). Towards a definition of inorganic nanoparticles from an environmental, health and safety

REFERENCES

perspective. In *Nature Nanotechnology* (Vol. 4, Issue 10, pp. 634–641). Nature Publishing Group. https://doi.org/10.1038/nnano.2009.242

Auwerx, J. (1991). The human leukemia cell line, THP-1: A multifacetted model for the study of monocyte-macrophage differentiation. *Experientia*, *47*(1), 22–31. https://doi.org/10.1007/BF02041244

Bai, C. L., Wang, D., Luan, Y. L., Huang, S. N., Liu, L. Y., & Guo, Y. (2024). A review on micro- and nanoplastics in humans: Implication for their translocation of barriers and potential health effects. In *Chemosphere* (Vol. 361). Elsevier Ltd. https://doi.org/10.1016/j.chemosphere.2024.142424

Banerjee, A., Billey, L. O., McGarvey, A. M., & Shelver, W. L. (2022). Effects of polystyrene micro/nanoplastics on liver cells based on particle size, surface functionalization, concentration and exposure period. *Science of the Total Environment*, *836*. https://doi.org/10.1016/j.scitotenv.2022.155621

Barnes, D. K. A., Galgani, F., Thompson, R. C., & Barlaz, M. (2009). Accumulation and fragmentation of plastic debris in global environments. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1526), 1985–1998. https://doi.org/10.1098/rstb.2008.0205

Bashirova, N., Poppitz, D., Klüver, N., Scholz, S., Matysik, J., & Alia, A. (2023). A mechanistic understanding of the effects of polyethylene terephthalate nanoplastics in the zebrafish (Danio rerio) embryo. *Scientific Reports*, *13*(1). https://doi.org/10.1038/s41598-023-28712-y

Beaumont, N. J., Aanesen, M., Austen, M. C., Börger, T., Clark, J. R., Cole, M., Hooper, T., Lindeque, P. K., Pascoe, C., & Wyles, K. J. (2019). Global ecological, social and economic impacts of marine plastic. *Marine Pollution Bulletin*, *142*, 189–195. https://doi.org/10.1016/j.marpolbul.2019.03.022

Belchamber, K. B. R., Singh, R., Batista, C. M., Whyte, M. K., Dockrell, D. H., Kilty, I., Robinson, M. J., Wedzicha, J. A., Barnes, P. J., & Donnelly, L. E. (2019). Defective bacterial phagocytosis is associated with dysfunctional mitochondria in COPD macrophages. *European Respiratory Journal*, *54*(4). https://doi.org/10.1183/13993003.02244-2018

Bellasi, A., Binda, G., Pozzi, A., Galafassi, S., Volta, P., & Bettinetti, R. (2020). Microplastic contamination in freshwater environments: A review, focusing on interactions with sediments and benthic organisms. In *Environments - MDPI* (Vol. 7, Issue 4). MDPI AG. https://doi.org/10.3390/environments7040030

Bikiaris, N., Nikolaidis, N. F., & Barmpalexis, P. (2024). Microplastics (MPs) in Cosmetics: A Review on Their Presence in Personal-Care, Cosmetic, and Cleaning Products (PCCPs) and Sustainable Alternatives from Biobased and Biodegradable Polymers. *Cosmetics*, *11*(5), 145. https://doi.org/10.3390/cosmetics11050145

Blackburn, K., & Green, D. (2022). The potential effects of microplastics on human health: What is known and what is unknown. *Ambio*, *51*(3), 518–530. https://doi.org/10.1007/s13280-021-01589-9

REFERENCES

Borrelle, S. B., Ringma, J., Law, K. L., Monnahan, C. C., Lebreton, L., McGivern, A., Murphy, E., Jambeck, J., Leonard, G. H., Hilleary, M. A., Eriksen, M., Possingham, H. P., De Frond, H., Gerber, L. R., Polidoro, B., Tahir, A., Bernard, M., Mallos, N., Barnes, M., & Rochman, C. M. (2020). Predicted growth in plastic waste exceeds efforts to mitigate plastic pollution. *Science*, *369*(6510), 1515–1518. https://doi.org/10.1126/science.aba3656

Brandts, I., Solà, R., Garcia-Ordoñez, M., Gella, A., Quintana, A., Martin, B., Esteve-Codina, A., Teles, M., & Roher, N. (2023a). Polystyrene nanoplastics target lysosomes interfering with lipid metabolism through the PPAR system and affecting macrophage functionalization. *Environmental Science: Nano*, *10*(9), 2245–2258. https://doi.org/10.1039/d2en01077a

Brandts, I., Solà, R., Garcia-Ordoñez, M., Gella, A., Quintana, A., Martin, B., Esteve-Codina, A., Teles, M., & Roher, N. (2023b). Polystyrene nanoplastics target lysosomes interfering with lipid metabolism through the PPAR system and affecting macrophage functionalization. *Environmental Science: Nano*, *10*(9), 2245–2258. https://doi.org/10.1039/d2en01077a

Browne, M. A., Crump, P., Niven, S. J., Teuten, E., Tonkin, A., Galloway, T., & Thompson, R. (2011). Accumulation of Microplastic on Shorelines Woldwide: Sources and Sinks. *Environmental Science & Technology*, *45*(21), 9175–9179. https://doi.org/10.1021/es201811s

Browne, M. A., Underwood, A. J., Chapman, M. G., Williams, R., Thompson, R. C., & Van Franeker, J. A. (2015). Linking effects of anthropogenic debris to ecological impacts. In *Proceedings of the Royal Society B: Biological Sciences* (Vol. 282, Issue 1807). Royal Society of London. https://doi.org/10.1098/rspb.2014.2929

Bruno, A., Dovizio, M., Milillo, C., Aruffo, E., Pesce, M., Gatta, M., Chiacchiaretta, P., Di Carlo, P., & Ballerini, P. (2024). Orally Ingested Micro- and Nano-Plastics: A Hidden Driver of Inflammatory Bowel Disease and Colorectal Cancer. In *Cancers* (Vol. 16, Issue 17). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/cancers16173079

Busch, M., Brouwer, H., Aalderink, G., Bredeck, G., Kämpfer, A. A. M., Schins, R. P. F., & Bouwmeester, H. (2023). Investigating nanoplastics toxicity using advanced stem cell-based intestinal and lung in vitro models. In *Frontiers in Toxicology* (Vol. 5). Frontiers Media S.A. https://doi.org/10.3389/ftox.2023.1112212

Carpenter, E., & Wolverton, S. (2017). Plastic litter in streams: The behavioral archaeology of a pervasive environmental problem. *Applied Geography*, *84*, 93–101. https://doi.org/https://doi.org/10.1016/j.apgeog.2017.04.010

Cary, C. M., DeLoid, G. M., Yang, Z., Bitounis, D., Polunas, M., Goedken, M. J., Buckley, B., Cheatham, B., Stapleton, P. A., & Demokritou, P. (2023). Ingested Polystyrene Nanospheres Translocate to Placenta and Fetal Tissues in Pregnant Rats: Potential Health Implications. *Nanomaterials*, *13*(4). https://doi.org/10.3390/nano13040720

Catarino, A. I., Frutos, A., & Henry, T. B. (2019). Use of fluorescent-labelled nanoplastics (NPs) to demonstrate NP absorption is inconclusive without adequate controls. *Science of the Total Environment*, *670*, 915–920. https://doi.org/10.1016/j.scitotenv.2019.03.194

REFERENCES

Chaudhari, S., Parmar, H., & Samnani, P. (2024). Microplastics in Personal Care Products and Cosmetics. In A. Kumar & V. Singh (Eds.), *Microplastics Pollution and its Remediation* (pp. 171–188). Springer Nature Singapore. https://doi.org/10.1007/978-981-97-4068-0_9

Chen, J., Yan, L., Zhang, Y., Liu, X., Wei, Y., Zhao, Y., Li, K., Shi, Y., Liu, H., Lai, W., Tian, L., & Lin, B. (2024). Maternal exposure to nanopolystyrene induces neurotoxicity in offspring through P53-mediated ferritinophagy and ferroptosis in the rat hippocampus. *Journal of Nanobiotechnology*, *22*(1), 651. https://doi.org/10.1186/s12951-024-02911-9

Chen, Q., Lin, L., Xu, Q., Tong, C., Li, M., Wang, Y., Zhu, Y., Zhao, Z., & Ge, R. S. (2022). Effect of triadimefon on rat placental morphology, function, and gene expression. *Toxicology Letters*, *371*, 25–37. https://doi.org/10.1016/j.toxlet.2022.09.009

Cheng, Y., Yang, Y., Bai, L., & Cui, J. (2024). Microplastics: an often-overlooked issue in the transition from chronic inflammation to cancer. *Journal of Translational Medicine*, *22*(1), 959. https://doi.org/10.1186/s12967-024-05731-5

Choe, S., Kim, Y., Won, Y., & Myung, J. (2021). Bridging Three Gaps in Biodegradable Plastics: Misconceptions and Truths About Biodegradation. In *Frontiers in Chemistry* (Vol. 9). Frontiers Media S.A. https://doi.org/10.3389/fchem.2021.671750

Choi, H., Kaneko, S., Suzuki, Y., Inamura, K., Nishikawa, M., & Sakai, Y. (2024). Size-Dependent Internalization of Microplastics and Nanoplastics Using In Vitro Model of the Human Intestine—Contribution of Each Cell in the Tri-Culture Models. *Nanomaterials*, *14*(17). https://doi.org/10.3390/nano14171435

Ciaglia, E., Montella, F., Trucillo, P., Ciardulli, M. C., Di Pietro, P., Amodio, G., Remondelli, P., Vecchione, C., Reverchon, E., Maffulli, N., Puca, A. A., & Della Porta, G. (2019). A bioavailability study on microbeads and nanoliposomes fabricated by dense carbon dioxide technologies using human-primary monocytes and flow cytometry assay. *International Journal of Pharmaceutics*, *570*. https://doi.org/10.1016/j.ijpharm.2019.118686

Collin-Faure, V., Dalzon, B., Devcic, J., Diemer, H., Cianférani, S., & Rabilloud, T. (2022). Does size matter? A proteomics-informed comparison of the effects of polystyrene beads of different sizes on macrophages. *Environmental Science: Nano*, *9*(8), 2827–2840. https://doi.org/10.1039/d2en00214k

Cortés, C., Domenech, J., Salazar, M., Pastor, S., Marcos, R., & Hernández, A. (2020). Nanoplastics as a potential environmental health factor: Effects of polystyrene nanoparticles on human intestinal epithelial Caco-2 cells. *Environmental Science: Nano*, *7*(1), 272–285. https://doi.org/10.1039/c9en00523d

Cox, K. D., Covernton, G. A., Davies, H. L., Dower, J. F., Juanes, F., & Dudas, S. E. (2019). Human Consumption of Microplastics. *Environmental Science & Technology*, *53*(12), 7068–7074. https://doi.org/10.1021/acs.est.9b01517

D Manikantha and Tulasi, C. D. S. L. N. and C. K. (2023). Cellular and Animal Toxicities of Micro- and Nanoplastics. In K. V. and R. P. Maddela Naga Raju and Reddy (Ed.), *Micro and Nanoplastics in Soil: Threats to Plant-Based Food* (pp. 261–292). Springer International Publishing. https://doi.org/10.1007/978-3-031-21195-9_12

REFERENCES

da Costa, J. P. (2018). Micro- and nanoplastics in the environment: Research and policymaking. In *Current Opinion in Environmental Science and Health* (Vol. 1, pp. 12–16). Elsevier B.V. https://doi.org/10.1016/j.coesh.2017.11.002

Daigneault, M., Preston, J. A., Marriott, H. M., Whyte, M. K. B., & Dockrell, D. H. (2010). The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS ONE*, *5*(1). https://doi.org/10.1371/journal.pone.0008668

Dayem, A. A., Hossain, M. K., Lee, S. Bin, Kim, K., Saha, S. K., Yang, G. M., Choi, H. Y., & Cho, S. G. (2017). The role of reactive oxygen species (ROS) in the biological activities of metallic nanoparticles. In *International Journal of Molecular Sciences* (Vol. 18, Issue 1). MDPI AG. https://doi.org/10.3390/ijms18010120

De Gaetano, A., Solodka, K., Zanini, G., Selleri, V., Mattioli, A. V., Nasi, M., & Pinti, M. (2021). Molecular mechanisms of mtdna-mediated inflammation. In *Cells* (Vol. 10, Issue 11). MDPI. https://doi.org/10.3390/cells10112898

de Thé, G. (2000). Epstein-Barr Virus and Burkitt's Lymphoma. In J. J. Goedert (Ed.), *Infectious Causes of Cancer: Targets for Intervention* (pp. 77–92). Humana Press. https://doi.org/10.1007/978-1-59259-024-7_5

Deng, L., Jian, Z., Xu, T., Li, F., Deng, H., Zhou, Y., Lai, S., Xu, Z., & Zhu, L. (2023). Macrophage Polarization: An Important Candidate Regulator for Lung Diseases. In *Molecules* (Vol. 28, Issue 5). MDPI. https://doi.org/10.3390/molecules28052379

Desidery, L., & Lanotte, M. (2022). 1 - Polymers and plastics: Types, properties, and manufacturing. In F. Giustozzi & S. Nizamuddin (Eds.), *Plastic Waste for Sustainable Asphalt Roads* (pp. 3–28). Woodhead Publishing. https://doi.org/https://doi.org/10.1016/B978-0-323-85789-5.00001-0

Deville, S., Honrath, B., Tran, Q. T. D., Fejer, G., Lambrichts, I., Nelissen, I., Dolga, A. M., & Salvati, A. (2020). Time-resolved characterization of the mechanisms of toxicity induced by silica and amino-modified polystyrene on alveolar-like macrophages. *Archives of Toxicology*, *94*(1), 173–186. https://doi.org/10.1007/s00204-019-02604-5

Dhaka, V., Singh, S., Anil, A. G., Sunil Kumar Naik, T. S., Garg, S., Samuel, J., Kumar, M., Ramamurthy, P. C., & Singh, J. (2022). Occurrence, toxicity and remediation of polyethylene terephthalate plastics. A review. In *Environmental Chemistry Letters* (Vol. 20, Issue 3, pp. 1777–1800). Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s10311-021-01384-8

Di, J., Reck, B. K., Miatto, A., & Graedel, T. E. (2021). United States plastics: Large flows, short lifetimes, and negligible recycling. *Resources, Conservation and Recycling*, *167*, 105440. https://doi.org/https://doi.org/10.1016/j.resconrec.2021.105440

Domenech, J., Hernández, A., Rubio, L., Marcos, R., & Cortés, C. (2020). Interactions of polystyrene nanoplastics with in vitro models of the human intestinal barrier. *Archives of Toxicology*, *94*(9), 2997–3012. https://doi.org/10.1007/s00204-020-02805-3

REFERENCES

Dris, R., Gasperi, J., Saad, M., Mirande, C., & Tassin, B. (2016). Synthetic fibers in atmospheric fallout: A source of microplastics in the environment? *Marine Pollution Bulletin*, *104*(1–2), 290–293. https://doi.org/10.1016/j.marpolbul.2016.01.006

Enyoh, C. E., Devi, A., Kadono, H., Wang, Q., & Rabin, M. H. (2023). The Plastic Within: Microplastics Invading Human Organs and Bodily Fluids Systems. In *Environments - MDPI* (Vol. 10, Issue 11). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/environments10110194

Enyoh, C. E., Verla, A. W., Verla, E. N., Ibe, F. C., & Amaobi, C. E. (2019). Airborne microplastics: a review study on method for analysis, occurrence, movement and risks. *Environmental Monitoring and Assessment*, *191*(11), 668. https://doi.org/10.1007/s10661-019-7842-0

Eriksen, M., Lebreton, L. C. M., Carson, H. S., Thiel, M., Moore, C. J., Borerro, J. C., Galgani, F., Ryan, P. G., & Reisser, J. (2014). Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. *PLoS ONE*, *9*(12). https://doi.org/10.1371/journal.pone.0111913

Fan, E. K. Y., & Fan, J. (2018). Regulation of alveolar macrophage death in acute lung inflammation. In *Respiratory Research* (Vol. 19, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s12931-018-0756-5

Fan, X., Xu, T., Peng, L., Tang, X., Chi, Q., Li, Z., Shi, G., & Li, S. (2022). *Polystyrene nanoplastic exposure enhances LPS-induced lung inammation in mice by inducing M1 polarization of macrophages via ROS/JAK/STAT signaling pathway*. https://doi.org/10.21203/rs.3.rs-1907903/v1

Feng, Y., Tu, C., Li, R., Wu, D., Yang, J., Xia, Y., Peijnenburg, W. J. G. M., & Luo, Y. (2023). A systematic review of the impacts of exposure to micro- and nano-plastics on human tissue accumulation and health. *Eco-Environment & Health*, *2*(4), 195–207. https://doi.org/https://doi.org/10.1016/j.eehl.2023.08.002

Fessler, M. B., & Summer, R. S. (2016). Surfactant lipids at the host-environment interface metabolic sensors, suppressors, and effectors of inflammatory lung disease. In *American Journal of Respiratory Cell and Molecular Biology* (Vol. 54, Issue 5, pp. 624–635). American Thoracic Society. https://doi.org/10.1165/rcmb.2016-0011PS

Gangadoo, S., Owen, S., Rajapaksha, P., Plaisted, K., Cheeseman, S., Haddara, H., Truong, V. K., Ngo, S. T., Vu, V. V, Cozzolino, D., Elbourne, A., Crawford, R., Latham, K., & Chapman, J. (2020). Nano-plastics and their analytical characterisation and fate in the marine environment: From source to sea. *Science of The Total Environment*, *732*, 138792. https://doi.org/https://doi.org/10.1016/j.scitotenv.2020.138792

Gautam Bhanu Pratap Singh and Qureshi, A. and G. A. and K. V. and G. M. (2024). Global Scenario of Plastic Production, Consumption, and Waste Generation and Their Impacts on Environment and Human Health. In P. and S. D. C. and G. R. Soni Ravindra and Debbarma (Ed.), *Advanced Strategies for Biodegradation of Plastic Polymers* (pp. 1–34). Springer Nature Switzerland. https://doi.org/10.1007/978-3-031-55661-6_1

REFERENCES

Gettings, S. M., Timbury, W., Dmochowska, A., Sharma, R., McGonigle, R., MacKenzie, L. E., Miquelard-Garnier, G., & Bourbia, N. (2024). Polyethylene terephthalate (PET) micro- and nanoplastic particles affect the mitochondrial efficiency of human brain vascular pericytes without inducing oxidative stress. *NanoImpact*, *34*. https://doi.org/10.1016/j.impact.2024.100508

Geyer, R., Jambeck, J. R., & Law, K. L. (2017). *Production, use, and fate of all plastics ever made*.

Geyer, R., Jambeck, J. R., & Law, K. L. (2024). Production, use, and fate of all plastics ever made. *Science Advances*, *3*(7), e1700782. https://doi.org/10.1126/sciadv.1700782

Gigault, J., El Hadri, H., Nguyen, B., Grassl, B., Rowenczyk, L., Tufenkji, N., Feng, S., & Wiesner, M. (2021). Nanoplastics are neither microplastics nor engineered nanoparticles. *Nature Nanotechnology*, *16*(5), 501–507. https://doi.org/10.1038/s41565-021-00886-4

Gigault, J., ter Halle, A., Baudrimont, M., Pascal, P.-Y., Gauffre, F., Phi, T.-L., El Hadri, H., Grassl, B., & Reynaud, S. (2018). Current opinion: What is a nanoplastic? *Environmental Pollution*, *235*, 1030–1034. https://doi.org/https://doi.org/10.1016/j.envpol.2018.01.024

Godderis, L., Thomas, R., Hubbard, A. E., Tabish, A. M., Hoet, P., Zhang, L., Smith, M. T., Veulemans, H., & McHale, C. M. (2012). Effect of chemical mutagens and carcinogens on gene expression profiles in human TK6 cells. *PLoS ONE*, *7*(6). https://doi.org/10.1371/journal.pone.0039205

Gonzalez, L., Lison, D., & Kirsch-Volders, M. (2008). Genotoxicity of engineered nanomaterials: A critical review. *Nanotoxicology*, *2*(4), 252–273. https://doi.org/10.1080/17435390802464986

Greven, A., Merk, T., Karagöz, F., Mohr, K., Klapper, M., Jovanović, B., & Palić, D. (2016). Polycarbonate and polystyrene nanoplastic particles act as stressors to the innate immune system of fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry*, *35*(12), 3093–3100. https://doi.org/10.1002/etc.3501

Guan, Q., Jiang, J., Huang, Y., Wang, Q., Liu, Z., Ma, X., Yang, X., Li, Y., Wang, S., Cui, W., Tang, J., Wan, H., Xu, Q., Tu, Y., Wu, D., & Xia, Y. (2023). The landscape of micron-scale particles including microplastics in human enclosed body fluids. *Journal of Hazardous Materials*, *442*, 130138. https://doi.org/https://doi.org/10.1016/j.jhazmat.2022.130138

Guan, S., Tao, S., Huang, Y., Jin, Y., Hu, Y., & Lu, J. (2020). Combined toxic effects of CBNPs and Pb on rat alveolar macrophage apoptosis and autophagy flux. *Ecotoxicology and Environmental Safety*, *205*, 111062. https://doi.org/https://doi.org/10.1016/j.ecoenv.2020.111062

Handbook of Polymer Applications in Medicine and Medical Devices. (2014). Elsevier. https://doi.org/10.1016/C2012-0-07906-9

Hartmann, N. B., Hüffer, T., Thompson, R. C., Hassellöv, M., Verschoor, A., Daugaard, A. E., Rist, S., Karlsson, T., Brennholt, N., Cole, M., Herrling, M. P., Hess, M. C., Ivleva, N. P., Lusher, A. L., & Wagner, M. (2019). Are We Speaking the Same Language? Recommendations for a Definition and Categorization Framework for Plastic Debris.

REFERENCES

Environmental Science & Technology, *53*(3), 1039–1047. https://doi.org/10.1021/acs.est.8b05297

Harusato, A., Seo, W., Abo, H., Nakanishi, Y., Nishikawa, H., & Itoh, Y. (2023). Impact of particulate microplastics generated from polyethylene terephthalate on gut pathology and immune microenvironments. *IScience*, *26*(4). https://doi.org/10.1016/j.isci.2023.106474

Herold, C. M. A. N. D. S. M. A. A. N. D. N. L. A. N. D. U. M. A. N. D. S. (2023). Alveolar macrophages in tissue homeostasis, inflammation, and infection: evolving concepts of therapeutic targeting. *The Journal of Clinical Investigation*, *133*(19). https://doi.org/10.1172/JCI170501

Hirt, N., & Body-Malapel, M. (2020a). Immunotoxicity and intestinal effects of nano- and microplastics: a review of the literature. *Particle and Fibre Toxicology*, *17*(1), 57. https://doi.org/10.1186/s12989-020-00387-7

Hirt, N., & Body-Malapel, M. (2020b). Immunotoxicity and intestinal effects of nano- and microplastics: a review of the literature. *Particle and Fibre Toxicology*, *17*(1), 57. https://doi.org/10.1186/s12989-020-00387-7

Horton, A. A., Walton, A., Spurgeon, D. J., Lahive, E., & Svendsen, C. (2017). Microplastics in freshwater and terrestrial environments: Evaluating the current understanding to identify the knowledge gaps and future research priorities. *Science of The Total Environment*, *586*, 127–141. https://doi.org/https://doi.org/10.1016/j.scitotenv.2017.01.190

Hossain, M. T., Shahid, M. A., Mahmud, N., Habib, A., Rana, M. M., Khan, S. A., & Hossain, M. D. (2024). Research and application of polypropylene: a review. In *Discover Nano* (Vol. 19, Issue 1). Springer. https://doi.org/10.1186/s11671-023-03952-z

Hua, X., & Wang, D. (2022). Cellular Uptake, Transport, and Organelle Response After Exposure to Microplastics and Nanoplastics: Current Knowledge and Perspectives for Environmental and Health Risks. *Reviews of Environmental Contamination and Toxicology*, *260*(1), 12. https://doi.org/10.1007/s44169-022-00013-x

Huang, J., Sun, X., Wang, Y., Su, J., Li, G., Wang, X., Yang, Y., Zhang, Y., Li, B., Zhang, G., Li, J., Du, J., Nanjundappa, R. H., Umeshappa, C. S., & Shao, K. (2023). Biological interactions of polystyrene nanoplastics: Their cytotoxic and immunotoxic effects on the hepatic and enteric systems. *Ecotoxicology and Environmental Safety*, *264*. https://doi.org/10.1016/j.ecoenv.2023.115447

Hutter, H.-P., Weitensfelder, L., & Poteser, M. (2024). Microplastics: Omnipresent and an ongoing challenge for medical science. *Wiener Klinische Wochenschrift*. https://doi.org/10.1007/s00508-024-02375-9

Ivleva, N. P. (2021). Chemical Analysis of Microplastics and Nanoplastics: Challenges, Advanced Methods, and Perspectives. In *Chemical Reviews* (Vol. 121, Issue 19, pp. 11886–11936). American Chemical Society. https://doi.org/10.1021/acs.chemrev.1c00178

Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., Narayan, R., & Law, K. L. (2015). Plastic waste inputs from land into the ocean. *Science*, *347*(6223), 768–771. https://doi.org/10.1126/science.1260352

REFERENCES

Jarvie, H. D. P. and K. Stephen. (2024, August 31). *nanoparticle*. Encyclopedia Britannica.

Jemec, A., Horvat, P., Kunej, U., Bele, M., & Kržan, A. (2016). Uptake and effects of microplastic textile fibers on freshwater crustacean Daphnia magna. *Environmental Pollution*, *219*, 201–209. https://doi.org/10.1016/j.envpol.2016.10.037

Jenner, L. C., Rotchell, J. M., Bennett, R. T., Cowen, M., Tentzeris, V., & Sadofsky, L. R. (2022). Detection of microplastics in human lung tissue using μFTIR spectroscopy. *Science of the Total Environment*, *831*. https://doi.org/10.1016/j.scitotenv.2022.154907

Jiang, W., Liu, Y., Wu, Y., Zhang, L., Zhang, B., Zhou, S., Zhang, P., Xu, T., Wu, M., & Lv, S. (2024). Polystyrene nanoplastics of different particle sizes regulate the polarization of pro-inflammatory macrophages. *Scientific Reports*, *14*(1). https://doi.org/10.1038/s41598-024-67289-y

Jin, Y., Qiu, J., Zhang, L., & Zhu, M. (2023). Biodegradation of polyethylene terephthalate: a review. In *Shengwu Gongcheng Xuebao/Chinese Journal of Biotechnology* (Vol. 39, Issue 11, pp. 4445–4462). Chinese Academy of Sciences. https://doi.org/10.13345/j.cjb.230215

Kaandorp, M. L. A., Lobelle, D., Kehl, C., Dijkstra, H. A., & van Sebille, E. (2023). Global mass of buoyant marine plastics dominated by large long-lived debris. *Nature Geoscience*, *16*(8), 689–694. https://doi.org/10.1038/s41561-023-01216-0

Kannan, K., & Vimalkumar, K. (2021). A Review of Human Exposure to Microplastics and Insights Into Microplastics as Obesogens. In *Frontiers in Endocrinology* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fendo.2021.724989

Karali, N., Khanna, N., & Shah, N. (n.d.). *Climate Impact of Primary Plastic Production Executive summary*.

Karami, A., Golieskardi, A., Keong Choo, C., Larat, V., Galloway, T. S., & Salamatinia, B. (2017). The presence of microplastics in commercial salts from different countries. *Scientific Reports*, *7*(1), 46173. https://doi.org/10.1038/srep46173

Karbalaei, S., Hanachi, P., Walker, T. R., & Cole, M. (2018). Occurrence, sources, human health impacts and mitigation of microplastic pollution. *Environmental Science and Pollution Research*, *25*(36), 36046–36063. https://doi.org/10.1007/s11356-018-3508-7

Kawasaki, T., & Kawai, T. (2014). Toll-like receptor signaling pathways. In *Frontiers in Immunology* (Vol. 5, Issue SEP). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2014.00461

Keerthana Devi, M., Karmegam, N., Manikandan, S., Subbaiya, R., Song, H., Kwon, E. E., Sarkar, B., Bolan, N., Kim, W., Rinklebe, J., & Govarthanan, M. (2022). Removal of nanoplastics in water treatment processes: A review. In *Science of the Total Environment* (Vol. 845). Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2022.157168

Kefer, S., Miesbauer, O., & Langowski, H. C. (2021). Environmental microplastic particles vs. Engineered plastic microparticles—a comparative review. In *Polymers* (Vol. 13, Issue 17). MDPI. https://doi.org/10.3390/polym13172881

REFERENCES

Kettler, K., Veltman, K., Van de meent, D., Wezel, A., & Hendriks, J. (2014). Cellular uptake of nanoparticles as determined by particle properties, experimental conditions, and cell type. *Environmental Toxicology and Chemistry / SETAC*, *33*. https://doi.org/10.1002/etc.2470

Kik, K., Bukowska, B., & Sicińska, P. (2020). Polystyrene nanoparticles: Sources, occurrence in the environment, distribution in tissues, accumulation and toxicity to various organisms. *Environmental Pollution*, *262*, 114297. https://doi.org/https://doi.org/10.1016/j.envpol.2020.114297

Klein, G., Giovanellat, B. C., Lindahl, T., Fialkowt, P. J., Singh, S., & Stehlint, J. S. (1974). *Direct Evidence for the Presence of Epstein-Barr Virus DNA and Nuclear Antigen in Malignant Epithelial Cells from Patients with Poorly Differentiated Carcinoma of the Nasopharynx (nude mice/transplantation/nucleic acid hybridization/isozyme)* (Vol. 71, Issue 12).

Korns, D., Frasch, S. C., Fernandez-Boyanapalli, R., Henson, P. M., & Bratton, D. L. (2011). Modulation of macrophage efferocytosis in inflammation. In *Frontiers in Immunology* (Vol. 2, Issue NOV). https://doi.org/10.3389/fimmu.2011.00057

Kosuth, M., Mason, S. A., & Wattenberg, E. V. (2018). Anthropogenic contamination of tap water, beer, and sea salt. *PLOS ONE*, *13*(4), e0194970-. https://doi.org/10.1371/journal.pone.0194970

Kumar, D., Biswas, J. K., Mulla, S. I., Singh, R., Shukla, R., Ahanger, M. A., Shekhawat, G. S., Verma, K. K., Siddiqui, M. W., & Seth, C. S. (2024). Micro and nanoplastics pollution: Sources, distribution, uptake in plants, toxicological effects, and innovative remediation strategies for environmental sustainability. *Plant Physiology and Biochemistry*, *213*, 108795. https://doi.org/https://doi.org/10.1016/j.plaphy.2024.108795

Latroche, C., Weiss-Gayet, M., Gitiaux, C., & Chazaud, B. (2018). Cell sorting of various cell types from mouse and human skeletal muscle. *Methods*, *134–135*, 50–55. https://doi.org/10.1016/j.ymeth.2017.12.013

Lau, W. W. Y., Shiran, Y., Bailey, R. M., Cook, E., Stuchtey, M. R., Koskella, J., Velis, C. A., Godfrey, L., Boucher, J., Murphy, M. B., Thompson, R. C., Jankowska, E., Castillo, A. C., Pilditch, T. D., Dixon, B., Koerselman, L., Kosior, E., Favoino, E., Gutberlet, J., … Palardy, J. E. (2020). Evaluating scenarios toward zero plastic pollution. *Science*, *369*(6510), 1455–1461. https://doi.org/10.1126/science.aba9475

Law, K. L., Sobkowicz, M. J., Shaver, M. P., & Hahn, M. E. (2024). Untangling the chemical complexity of plastics to improve life cycle outcomes. *Nature Reviews Materials*, *9*(9), 657–667. https://doi.org/10.1038/s41578-024-00705-x

Lee, H., Song, S. J., Kim, C.-S., & Park, B. (2024). Polystyrene nanoplastics-induced intestinal barrier disruption via inflammation and apoptosis in zebrafish larvae (Danio Rerio). *Aquatic Toxicology*, *274*, 107027. https://doi.org/https://doi.org/10.1016/j.aquatox.2024.107027

Leslie, H. A., van Velzen, M. J. M., Brandsma, S. H., Vethaak, A. D., Garcia-Vallejo, J. J., & Lamoree, M. H. (2022). Discovery and quantification of plastic particle pollution in human blood. *Environment International*, *163*, 107199. https://doi.org/https://doi.org/10.1016/j.envint.2022.107199

REFERENCES

Lesniak, A., Salvati, A., Santos-Martinez, M. J., Radomski, M. W., Dawson, K. A., & Åberg, C. (2013). Nanoparticle Adhesion to the Cell Membrane and Its Effect on Nanoparticle Uptake Efficiency. *Journal of the American Chemical Society*, *135*(4), 1438–1444. https://doi.org/10.1021/ja309812z

Lett, Z., Hall, A., Skidmore, S., & Alves, N. J. (2021). Environmental microplastic and nanoplastic: Exposure routes and effects on coagulation and the cardiovascular system. *Environmental Pollution*, *291*, 118190. https://doi.org/https://doi.org/10.1016/j.envpol.2021.118190

Li, L., Li, S., Xu, Y., Ren, L., Yang, L., Liu, X., Dai, Y., Zhao, J., & Yue, T. (2022a). Distinguishing the nanoplastic–cell membrane interface by polymer type and aging properties: : translocation, transformation and perturbation. *Environmental Science: Nano*. https://api.semanticscholar.org/CorpusID:253789447

Li, L., Li, S., Xu, Y., Ren, L., Yang, L., Liu, X., Dai, Y., Zhao, J., & Yue, T. (2022b). Distinguishing the nanoplastic–cell membrane interface by polymer type and aging properties: : translocation, transformation and perturbation. *Environmental Science: Nano*. https://api.semanticscholar.org/CorpusID:253789447

Li, L., Li, S., Xu, Y., Ren, L., Yang, L., Liu, X., Dai, Y., Zhao, J., & Yue, T. (2023). Distinguishing the nanoplastic–cell membrane interface by polymer type and aging properties: translocation, transformation and perturbation. *Environ. Sci.: Nano*, *10*(2), 440–453. https://doi.org/10.1039/D2EN00800A

Li, X., Chen, S., Guo, X., Wu, Q., Seo, J.-E., Guo, L., Manjanatha, M. G., Zhou, T., Witt, K. L., & Mei, N. (2020). Development and Application of TK6-derived Cells Expressing Human Cytochrome P450s for Genotoxicity Testing. *Toxicological Sciences*, *175*(2), 251–265. https://doi.org/10.1093/toxsci/kfaa035

Li, X., & Li, Z. (2024). Perspectives on the Toxic Effects of Micro- and Nanoplastics on the Environment: A Bibliometric Analysis of the 2014 to 2023 Period. *Toxics*, *12*(9). https://doi.org/10.3390/toxics12090676

Lim, D., Jeong, J., Song, K. S., Sung, J. H., Oh, S. M., & Choi, J. (2021). Inhalation toxicity of polystyrene micro(nano)plastics using modified OECD TG 412. *Chemosphere*, *262*. https://doi.org/10.1016/j.chemosphere.2020.128330

Liu, L., Du, R., Niu, L., Li, P., & Li, Z.-H. (2024). A Latest Review on Micro- and Nanoplastics in the Aquatic Environment: The Comparative Impact of Size on Environmental Behavior and Toxic Effect. *Bulletin of Environmental Contamination and Toxicology*, *112 2*, 36. https://api.semanticscholar.org/CorpusID:267657176

Liu, L., Xu, K., Zhang, B., Ye, Y., Zhang, Q., & Jiang, W. (2021). Cellular internalization and release of polystyrene microplastics and nanoplastics. *Science of the Total Environment*, *779*. https://doi.org/10.1016/j.scitotenv.2021.146523

Llorca, M., & Farré, M. (2021a). Current Insights into Potential Effects of Micro-Nanoplastics on Human Health by in-vitro Tests. In *Frontiers in Toxicology* (Vol. 3). Frontiers Media S.A. https://doi.org/10.3389/ftox.2021.752140

REFERENCES

Llorca, M., & Farré, M. (2021b). Current Insights into Potential Effects of Micro-Nanoplastics on Human Health by in-vitro Tests. In *Frontiers in Toxicology* (Vol. 3). Frontiers Media S.A. https://doi.org/10.3389/ftox.2021.752140

Llorca, M., & Farré, M. (2021c). Current Insights into Potential Effects of Micro-Nanoplastics on Human Health by in-vitro Tests. In *Frontiers in Toxicology* (Vol. 3). Frontiers Media S.A. https://doi.org/10.3389/ftox.2021.752140

Lu, Y.-Y., Li, H., Ren, H., Zhang, X., Huang, F., Zhang, D., Huang, Q., & Zhang, X. (2022). Size-dependent effects of polystyrene nanoplastics on autophagy response in human umbilical vein endothelial cells. *Journal of Hazardous Materials*, *421*, 126770. https://doi.org/https://doi.org/10.1016/j.jhazmat.2021.126770

Lugg, S. T., Scott, A., Parekh, D., Naidu, B., & Thickett, D. R. (2022). Cigarette smoke exposure and alveolar macrophages: mechanisms for lung disease. *Thorax*, *77*(1), 94–101. https://doi.org/10.1136/thoraxjnl-2020-216296

Magrì, D., Veronesi, M., Sánchez-Moreno, P., Tolardo, V., Bandiera, T., Pompa, P. P., Athanassiou, A., & Fragouli, D. (2021a). PET nanoplastics interactions with water contaminants and their impact on human cells. *Environmental Pollution*, *271*, 116262. https://doi.org/https://doi.org/10.1016/j.envpol.2020.116262

Magrì, D., Veronesi, M., Sánchez-Moreno, P., Tolardo, V., Bandiera, T., Pompa, P. P., Athanassiou, A., & Fragouli, D. (2021b). PET nanoplastics interactions with water contaminants and their impact on human cells. *Environmental Pollution*, *271*. https://doi.org/10.1016/j.envpol.2020.116262

Manke, A., Wang, L., & Rojanasakul, Y. (2013). Mechanisms of nanoparticle-induced oxidative stress and toxicity. In *BioMed Research International* (Vol. 2013). https://doi.org/10.1155/2013/942916

Mason, S. A., Welch, V. G., & Neratko, J. (2018). Synthetic Polymer Contamination in Bottled Water. *Frontiers in Chemistry*, *6*. https://doi.org/10.3389/fchem.2018.00407

Microplastic Contamination in Aquatic Environments. (2018). Elsevier. https://doi.org/10.1016/C2016-0-04784-8

Mohan Qin. (2024). *What are nanoplastics? An engineer explains concerns about particles too small to see*. The Conversation.

Mortensen, N. P., Fennell, T. R., & Johnson, L. M. (2021). Unintended human ingestion of nanoplastics and small microplastics through drinking water, beverages, and food sources. *NanoImpact*, *21*, 100302. https://doi.org/https://doi.org/10.1016/j.impact.2021.100302

Murphy, K., & Casey Weaver. (2016). *Janeway's immunobiology*. Garland science.

Murray, P. J. (2017). Macrophage Polarization. In *Annual Review of Physiology* (Vol. 79, pp. 541–566). Annual Reviews Inc. https://doi.org/10.1146/annurev-physiol-022516-034339

Namsheer, K., & Rout, C. S. (2021). Conducting polymers: a comprehensive review on recent advances in synthesis, properties and applications. In *RSC Advances* (Vol. 11, Issue 10, pp. 5659–5697). Royal Society of Chemistry. https://doi.org/10.1039/d0ra07800j

REFERENCES

NAPCOR. (2020). *What is PET?* NAPCOR.

Napper, I. E., Baroth, A., Barrett, A. C., Bhola, S., Chowdhury, G. W., Davies, B. F. R., Duncan, E. M., Kumar, S., Nelms, S. E., Hasan Niloy, M. N., Nishat, B., Maddalene, T., Thompson, R. C., & Koldewey, H. (2021). The abundance and characteristics of microplastics in surface water in the transboundary Ganges River. *Environmental Pollution*, *274*, 116348. https://doi.org/https://doi.org/10.1016/j.envpol.2020.116348

Nazeer, N., Bhargava, A., Soni, N., Tiwari, R., Ratre, P., & Mishra, P. K. (2024). Unravelling the molecular dimensions of atmospheric Micro(nano)Plastics: Exploring potential impacts on human health and strategies for detection. *Physics and Chemistry of the Earth, Parts A/B/C*, *135*, 103604. https://doi.org/10.1016/j.pce.2024.103604

Nizzetto, L., Langaas, S., & Futter, M. (2016). Pollution: Do microplastics spill on to farm soils? *Nature*, *537*(7621), 488. https://doi.org/10.1038/537488b

On the plastics crisis. (2023). *Nature Sustainability*, *6*(10), 1137. https://doi.org/10.1038/s41893-023-01236-z

Osman, A. I., Hosny, M., Eltaweil, A. S., Omar, S., Elgarahy, A. M., Farghali, M., Yap, P.-S., Wu, Y.-S., Nagandran, S., Batumalaie, K., Gopinath, S. C. B., John, O. D., Sekar, M., Saikia, T., Karunanithi, P., Hatta, M. H. M., & Akinyede, K. A. (2023). Microplastic sources, formation, toxicity and remediation: a review. *Environmental Chemistry Letters*, *21*(4), 2129–2169. https://doi.org/10.1007/s10311-023-01593-3

Otorkpa, O. J., & Otorkpa, C. O. (2024). Health effects of microplastics and nanoplastics: review of published case reports. *Environmental Analysis Health and Toxicology*, *39*(2), e2024020. https://doi.org/10.5620/eaht.2024020

Park, E. K., Jung, H. S., Yang, H. I., Yoo, M. C., Kim, C., & Kim, K. S. (2007). Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflammation Research*, *56*(1), 45–50. https://doi.org/10.1007/s00011-007-6115-5

Peng, Y., Zhou, M., Yang, H., Qu, R., Qiu, Y., Hao, J., Bi, H., & Guo, D. (2023). Regulatory Mechanism of M1/M2 Macrophage Polarization in the Development of Autoimmune Diseases. In *Mediators of Inflammation* (Vol. 2023). Hindawi Limited. https://doi.org/10.1155/2023/8821610

Pivotal fourth session of negotiations on a global plastics. (2024).

Plasticseurope. (2023). *Plastics Europe*.

Płuciennik, K., Sicińska, P., Misztal, W., & Bukowska, B. (2024). Important Factors Affecting Induction of Cell Death, Oxidative Stress and DNA Damage by Nano- and Microplastic Particles In Vitro. In *Cells* (Vol. 13, Issue 9). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/cells13090768

Polasky, C., Studt, T., Steuer, A.-K., Loyal, K., Lüdtke-Buzug, K., Bruchhage, K.-L., & Pries, R. (2022). Impact of Superparamagnetic Iron Oxide Nanoparticles on THP-1 Monocytes and Monocyte-Derived Macrophages. *Frontiers in Molecular Biosciences*, *9*. https://doi.org/10.3389/fmolb.2022.811116

REFERENCES

Pradel, A., Catrouillet, C., & Gigault, J. (2023). The environmental fate of nanoplastics: What we know and what we need to know about aggregation. *NanoImpact*, *29*, 100453. https://doi.org/https://doi.org/10.1016/j.impact.2023.100453

Prata, J. C. (2018). Airborne microplastics: Consequences to human health? *Environmental Pollution*, *234*, 115–126. https://doi.org/https://doi.org/10.1016/j.envpol.2017.11.043

Prata, J. C., da Costa, J. P., Lopes, I., Duarte, A. C., & Rocha-Santos, T. (2020). Environmental exposure to microplastics: An overview on possible human health effects. *Science of The Total Environment*, *702*, 134455. https://doi.org/https://doi.org/10.1016/j.scitotenv.2019.134455

Preda, O.-T., Vlasceanu, A.-M., Andreescu, C. V., Tsatsakis, A., Mezhuev, Y., Negrei, C., & Baconi, D. L. (2024). Health Implications of Widespread Micro- and Nanoplastic Exposure: Environmental Prevalence, Mechanisms, and Biological Impact on Humans. *Toxics*, *12*(10), 730. https://doi.org/10.3390/toxics12100730

Prüst, M., Meijer, J., & Westerink, R. H. S. (2020). The plastic brain: Neurotoxicity of micro- And nanoplastics. In *Particle and Fibre Toxicology* (Vol. 17, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s12989-020-00358-y

Pulskamp, K., Diabaté, S., & Krug, H. F. (2007). Carbon nanotubes show no sign of acute toxicity but induce intracellular reactive oxygen species in dependence on contaminants. *Toxicology Letters*, *168*(1), 58–74. https://doi.org/https://doi.org/10.1016/j.toxlet.2006.11.001

Pulvertaft, R. J. V. (1965). A study of malignant tumours in Nigeria by short-term tissue culture. *Journal of Clinical Pathology*, *18*(3), 261–273. https://doi.org/10.1136/jcp.18.3.261

Rahman, A., Sarkar, A., Yadav, O. P., Achari, G., & Slobodnik, J. (2021). Potential human health risks due to environmental exposure to nano- and microplastics and knowledge gaps: A scoping review. *Science of The Total Environment*, *757*, 143872. https://doi.org/https://doi.org/10.1016/j.scitotenv.2020.143872

Rai, M., Pant, G., Pant, K., Aloo, B. N., Kumar, G., Singh, H. B., & Tripathi, V. (2023). Microplastic Pollution in Terrestrial Ecosystems and Its Interaction with Other Soil Pollutants: A Potential Threat to Soil Ecosystem Sustainability. In *Resources* (Vol. 12, Issue 6). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/resources12060067

Ramirez Arenas, L., Ramseier Gentile, S., Zimmermann, S., & Stoll, S. (2022). Fate and removal efficiency of polystyrene nanoplastics in a pilot drinking water treatment plant. *Science of the Total Environment*, *813*. https://doi.org/10.1016/j.scitotenv.2021.152623

Ramsperger, A. F. R. M., Bergamaschi, E., Panizzolo, M., Fenoglio, I., Barbero, F., Peters, R., Undas, A., Purker, S., Giese, B., Lalyer, C. R., Tamargo, A., Moreno-Arribas, M. V., Grossart, H.-P., Kühnel, D., Dietrich, J., Paulsen, F., Afanou, A. K., Zienolddiny-Narui, S., Eriksen Hammer, S., … Laforsch, C. (2023). Nano- and microplastics: a comprehensive review on their exposure routes, translocation, and fate in humans. *NanoImpact*, *29*, 100441. https://doi.org/https://doi.org/10.1016/j.impact.2022.100441

REFERENCES

Revel, M., Châtel, A., & Mouneyrac, C. (2018). Micro(nano)plastics: A threat to human health? *Current Opinion in Environmental Science & Health*, *1*, 17–23. https://doi.org/https://doi.org/10.1016/j.coesh.2017.10.003

Rillig, M. C., & Lehmann, A. (2020). Microplastic in terrestrial ecosystems. *Science*, *368*(6498), 1430–1431. https://doi.org/10.1126/science.abb5979

Rios, L. M., Moore, C., & Jones, P. R. (2007). Persistent organic pollutants carried by synthetic polymers in the ocean environment. *Marine Pollution Bulletin*, *54*(8), 1230–1237. https://doi.org/https://doi.org/10.1016/j.marpolbul.2007.03.022

Rodrigues, A. C. B., de Jesus, G. P., Waked, D., Gomes, G. L., Silva, T. M., Yariwake, V. Y., da Silva, M. P., Magaldi, A. J., & Veras, M. M. (2022). Scientific Evidence about the Risks of Micro and Nanoplastics (MNPLs) to Human Health and Their Exposure Routes through the Environment. In *Toxics* (Vol. 10, Issue 6). MDPI. https://doi.org/10.3390/toxics10060308

Ruan, Y., Zhong, Z., Liu, X., Li, Z., Li, J., Sun, L., & Sen, H. (2023). Correlation between cellular uptake and cytotoxicity of polystyrene micro/nanoplastics in HeLa cells: A size-dependent matter. *PLoS ONE*, *18*(8 August). https://doi.org/10.1371/journal.pone.0289473

Rubio, L., Barguilla, I., Domenech, J., Marcos, R., & Hernández, A. (2020a). Biological effects, including oxidative stress and genotoxic damage, of polystyrene nanoparticles in different human hematopoietic cell lines. *Journal of Hazardous Materials*, *398*, 122900. https://doi.org/https://doi.org/10.1016/j.jhazmat.2020.122900

Rubio, L., Barguilla, I., Domenech, J., Marcos, R., & Hernández, A. (2020b). Biological effects, including oxidative stress and genotoxic damage, of polystyrene nanoparticles in different human hematopoietic cell lines. *Journal of Hazardous Materials*, *398*, 122900. https://doi.org/https://doi.org/10.1016/j.jhazmat.2020.122900

Saleh, A. F., Priestley, C. C., Gooderham, N. J., & Fellows, M. D. (2015). Re-evaluation of the mutagenic response to phosphorothioate nucleotides in human lymphoblastoid TK6 cells. *Toxicological Sciences*, *145*(1), 169–176. https://doi.org/10.1093/toxsci/kfv043

Salvia, R., Rico, L. G., Bradford, J. A., Ward, M. D., Olszowy, M. W., Martínez, C., Madrid-Aris, Á. D., Grífols, J. R., Ancochea, Á., Gomez-Muñoz, L., Vives-Pi, M., Martínez-Cáceres, E., Fernández, M. A., Sorigue, M., & Petriz, J. (2023). Fast-screening flow cytometry method for detecting nanoplastics in human peripheral blood. *MethodsX*, *10*, 102057. https://doi.org/https://doi.org/10.1016/j.mex.2023.102057

Santillo, D., Miller, K., & Johnston, P. (2017). Microplastics as contaminants in commercially important seafood species. *Integrated Environmental Assessment and Management*, *13*(3), 516–521. https://doi.org/10.1002/ieam.1909

Sarma, D. K., Dubey, R., Samarth, R. M., Shubham, S., Chowdhury, P., Kumawat, M., Verma, V., Tiwari, R. R., & Kumar, M. (2022). The Biological Effectsof Polystyrene Nanoplasticson Human Peripheral Blood Lymphocytes. *Nanomaterials*, *12*(10). https://doi.org/10.3390/nano12101632

Sawyer, L. C., Grubb, D. T., & Meyers, G. F. (2008). Polymer microscopy: Third edition. In *Polymer Microscopy: Third Edition*. Springer New York. https://doi.org/10.1007/978-0-387-72628-1

REFERENCES

Schirinzi, G. F., Pérez-Pomeda, I., Sanchís, J., Rossini, C., Farré, M., & Barceló, D. (2017). Cytotoxic effects of commonly used nanomaterials and microplastics on cerebral and epithelial human cells. *Environmental Research*, *159*, 579–587. https://doi.org/https://doi.org/10.1016/j.envres.2017.08.043

Schwarz, A. E., Ligthart, T. N., Boukris, E., & van Harmelen, T. (2019). Sources, transport, and accumulation of different types of plastic litter in aquatic environments: A review study. *Marine Pollution Bulletin*, *143*, 92–100. https://doi.org/https://doi.org/10.1016/j.marpolbul.2019.04.029

Shi, X., Wang, X., Huang, R., Tang, C., Hu, C., Ning, P., & Wang, F. (2022). Cytotoxicity and Genotoxicity of Polystyrene Micro-and Nanoplastics with Different Size and Surface Modification in A549 Cells. *International Journal of Nanomedicine*, *17*, 4509–4523. https://doi.org/10.2147/IJN.S381776

Singh, N., Manshian, B., Jenkins, G. J. S., Griffiths, S. M., Williams, P. M., Maffeis, T. G. G., Wright, C. J., & Doak, S. H. (2009). NanoGenotoxicology: The DNA damaging potential of engineered nanomaterials. *Biomaterials*, *30*(23), 3891–3914. https://doi.org/https://doi.org/10.1016/j.biomaterials.2009.04.009

Smart, D. J., Helbling, F. R., Verardo, M., Huber, A., McHugh, D., & Vanscheeuwijck, P. (2020). Development of an integrated assay in human TK6 cells to permit comprehensive genotoxicity analysis in vitro. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *849*, 503129. https://doi.org/https://doi.org/10.1016/j.mrgentox.2019.503129

Soliman, E., Elhassanny, A. E. M., Malur, A., McPeek, M., Bell, A., Leffler, N., Van Dross, R., Jones, J. L., Malur, A. G., & Thomassen, M. J. (2020). Impaired mitochondrial function of alveolar macrophages in carbon nanotube-induced chronic pulmonary granulomatous disease. *Toxicology*, *445*. https://doi.org/10.1016/j.tox.2020.152598

Stapleton, P. A. (2021). Microplastic and nanoplastic transfer, accumulation, and toxicity in humans. In *Current Opinion in Toxicology* (Vol. 28, pp. 62–69). Elsevier B.V. https://doi.org/10.1016/j.cotox.2021.10.001

Steven M Bryce 1, S. L. A. J. C. B. S. D. D. (2010). Miniaturized flow cytometry-based CHO-K1 micronucleus assay discriminates aneugenic and clastogenic modes of action. *Environmental and Molecular Mutagenesis*, *52*(4), 280–286.

Stock, V., Böhmert, L., Coban, G., Tyra, G., Vollbrecht, M.-L., Voss, L., Paul, M. B., Braeuning, A., & Sieg, H. (2022). Microplastics and nanoplastics: Size, surface and dispersant – What causes the effect? *Toxicology in Vitro*, *80*, 105314. https://doi.org/https://doi.org/10.1016/j.tiv.2022.105314

Stoian, A. I. (2024). The Impact of Microplastics and Nanoplastics on Human Digestive, Respiratory and Cardiovascular Systems. *International Conference KNOWLEDGE-BASED ORGANIZATION*, *30*(3), 1–7. https://doi.org/10.2478/kbo-2024-0097

Suaria, G., Avio, C. G., Mineo, A., Lattin, G. L., Magaldi, M. G., Belmonte, G., Moore, C. J., Regoli, F., & Aliani, S. (2016). The Mediterranean Plastic Soup: Synthetic polymers in Mediterranean surface waters. *Scientific Reports*, *6*. https://doi.org/10.1038/srep37551

REFERENCES

Summer, M., Ashraf, R., Ali, S., Bach, H., Noor, S., Noor, Q., Riaz, S., & Khan, R. R. M. (2024). Inflammatory response of nanoparticles: Mechanisms, consequences, and strategies for mitigation. *Chemosphere*, *363*, 142826. https://doi.org/https://doi.org/10.1016/j.chemosphere.2024.142826

Sun, A., & Wang, W.-X. (2023). Human Exposure to Microplastics and Its Associated Health Risks. *Environment & Health*, *1*(3), 139–149. https://doi.org/10.1021/envhealth.3c00053

Tapak, M., Sadeghi, S., Ghazanfari, T., & Mosaffa, N. (2023a). Chemical exposure and alveolar macrophages responses: "the role of pulmonary defense mechanism in inhalation injuries." In *BMJ Open Respiratory Research* (Vol. 10, Issue 1). BMJ Publishing Group. https://doi.org/10.1136/bmjresp-2022-001589

Tapak, M., Sadeghi, S., Ghazanfari, T., & Mosaffa, N. (2023b). Chemical exposure and alveolar macrophages responses: the role of pulmonary defense mechanism in inhalation injuries. *BMJ Open Respiratory Research*, *10*(1). https://doi.org/10.1136/bmjresp-2022-001589

Tardito, S., Martinelli, G., Soldano, S., Paolino, S., Pacini, G., Patane, M., Alessandri, E., Smith, V., & Cutolo, M. (2019). Macrophage M1/M2 polarization and rheumatoid arthritis: A systematic review. In *Autoimmunity Reviews* (Vol. 18, Issue 11). Elsevier B.V. https://doi.org/10.1016/j.autrev.2019.102397

Tenuta, T., Monopoli, M. P., Kim, J. A., Salvati, A., Dawson, K. A., Sandin, P., & Lynch, I. (2011). Elution of labile fluorescent dye from nanoparticles during biological use. *PLoS ONE*, *6*(10). https://doi.org/10.1371/journal.pone.0025556

Thornton Hampton, L. M., Lowman, H., Coffin, S., Darin, E., De Frond, H., Hermabessiere, L., Miller, E., de Ruijter, V. N., Faltynkova, A., Kotar, S., Monclús, L., Siddiqui, S., Völker, J., Brander, S., Koelmans, A. A., Rochman, C. M., Wagner, M., & Mehinto, A. C. (2022). A living tool for the continued exploration of microplastic toxicity. *Microplastics and Nanoplastics*, *2*(1). https://doi.org/10.1186/s43591-022-00032-4

Thushari, G. G. N., & Senevirathna, J. D. M. (2020). Plastic pollution in the marine environment. *Heliyon*, *6*(8), e04709. https://doi.org/https://doi.org/10.1016/j.heliyon.2020.e04709

Torres-Agullo, A., Karanasiou, A., Moreno, T., & Lacorte, S. (2022). Airborne microplastic particle concentrations and characterization in indoor urban microenvironments. *Environmental Pollution*, *308*, 119707. https://doi.org/https://doi.org/10.1016/j.envpol.2022.119707

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., & Tada, K. (1980). Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *International Journal of Cancer*, *26*(2), 171–176. https://doi.org/10.1002/ijc.2910260208

Urbanek, A. K., Kosiorowska, K. E., & Mirończuk, A. M. (2021). Current Knowledge on Polyethylene Terephthalate Degradation by Genetically Modified Microorganisms. In *Frontiers in Bioengineering and Biotechnology* (Vol. 9). Frontiers Media S.A. https://doi.org/10.3389/fbioe.2021.771133

Vance, M. E., Kuiken, T., Vejerano, E. P., McGinnis, S. P., Hochella, M. F., & Hull, D. R. (2015). Nanotechnology in the real world: Redeveloping the nanomaterial consumer products

REFERENCES

inventory. *Beilstein Journal of Nanotechnology*, *6*(1), 1769–1780. https://doi.org/10.3762/bjnano.6.181

Vandermeersch, G., Van Cauwenberghe, L., Janssen, C. R., Marques, A., Granby, K., Fait, G., Kotterman, M. J. J., Diogène, J., Bekaert, K., Robbens, J., & Devriese, L. (2015). A critical view on microplastic quantification in aquatic organisms. *Environmental Research*, *143*, 46–55. https://doi.org/https://doi.org/10.1016/j.envres.2015.07.016

Vasse, G. F., & Melgert, B. N. (2024). Microplastic and plastic pollution: impact on respiratory disease and health. *European Respiratory Review*, *33*(172), 230226. https://doi.org/10.1183/16000617.0226-2023

Vicki Contie. (2024). *Plastic particles in bottled water*.

Villacorta, A., Rubio, L., Alaraby, M., López-Mesas, M., Fuentes-Cebrian, V., Moriones, O. H., Marcos, R., & Hernández, A. (2022). A new source of representative secondary PET nanoplastics. Obtention, characterization, and hazard evaluation. *Journal of Hazardous Materials*, *439*. https://doi.org/10.1016/j.jhazmat.2022.129593

Wang, K., Du, Y., Li, P., Guan, C., Zhou, M., Wu, L., Liu, Z., & Huang, Z. (2024). Nanoplastics causes heart aging/myocardial cell senescence through the Ca2+/mtDNA/cGAS-STING signaling cascade. *Journal of Nanobiotechnology*, *22*(1). https://doi.org/10.1186/s12951-024-02375-x

Wang, X., Ren, X. M., He, H., Li, F., Liu, K., Zhao, F., Hu, H., Zhang, P., Huang, B., & Pan, X. (2023). Cytotoxicity and pro-inflammatory effect of polystyrene nano-plastic and micro-plastic on RAW264.7 cells. *Toxicology*, *484*. https://doi.org/10.1016/j.tox.2022.153391

Wei, K., Chen, T., Fang, H., Shen, X., Tang, Z., & Zhao, J. (2024). Mitochondrial DNA release via the mitochondrial permeability transition pore activates the cGAS-STING pathway, exacerbating inflammation in acute Kawasaki disease. *Cell Communication and Signaling*, *22*(1). https://doi.org/10.1186/s12964-024-01677-9

Whitfield, R., Truong, N. P., Messmer, D., Parkatzidis, K., Rolland, M., & Anastasaki, A. (2019). Tailoring polymer dispersity and shape of molecular weight distributions: methods and applications. *Chem. Sci.*, *10*(38), 8724–8734. https://doi.org/10.1039/C9SC03546J

Williams, A. T., & Rangel-Buitrago, N. (2022). The past, present, and future of plastic pollution. *Marine Pollution Bulletin*, *176*, 113429. https://doi.org/https://doi.org/10.1016/j.marpolbul.2022.113429

Wolff, C. M., Singer, D., Schmidt, A., & Bekeschus, S. (2023a). Immune and inflammatory responses of human macrophages, dendritic cells, and T-cells in presence of micro- and nanoplastic of different types and sizes. *Journal of Hazardous Materials*, *459*, 132194. https://doi.org/https://doi.org/10.1016/j.jhazmat.2023.132194

Wolff, C. M., Singer, D., Schmidt, A., & Bekeschus, S. (2023b). Immune and inflammatory responses of human macrophages, dendritic cells, and T-cells in presence of micro- and nanoplastic of different types and sizes. *Journal of Hazardous Materials*, *459*. https://doi.org/10.1016/j.jhazmat.2023.132194

REFERENCES

Wright, S. L., Ulke, J., Font, A., Chan, K. L. A., & Kelly, F. J. (2020). Atmospheric microplastic deposition in an urban environment and an evaluation of transport. *Environment International*, *136*, 105411. https://doi.org/https://doi.org/10.1016/j.envint.2019.105411

Wu, B., Wu, X., Liu, S., Wang, Z., & Chen, L. (2019a). Size-dependent effects of polystyrene microplastics on cytotoxicity and efflux pump inhibition in human Caco-2 cells. *Chemosphere*, *221*, 333–341. https://doi.org/https://doi.org/10.1016/j.chemosphere.2019.01.056

Wu, B., Wu, X., Liu, S., Wang, Z., & Chen, L. (2019b). Size-dependent effects of polystyrene microplastics on cytotoxicity and efflux pump inhibition in human Caco-2 cells. *Chemosphere*, *221*, 333–341. https://doi.org/10.1016/j.chemosphere.2019.01.056

Xayachak, T., Haque, N., Lau, D., & Pramanik, B. K. (2024). The missing link: A systematic review of microplastics and its neglected role in life-cycle assessment. *Science of The Total Environment*, *954*, 176513. https://doi.org/https://doi.org/10.1016/j.scitotenv.2024.176513

Xie, H., Tian, X., Lin, X., Chen, R., Hameed, S., Wang, L., Yu, Y.-L., Li, B., & Li, Y.-F. (2023). Nanoplastic-Induced Biological Effects In Vivo and In Vitro: An Overview. *Reviews of Environmental Contamination and Toxicology*, *261*(1), 2. https://doi.org/10.1007/s44169-023-00027-z

Yee, M. S. L., Hii, L. W., Looi, C. K., Lim, W. M., Wong, S. F., Kok, Y. Y., Tan, B. K., Wong, C. Y., & Leong, C. O. (2021). Impact of microplastics and nanoplastics on human health. In *Nanomaterials* (Vol. 11, Issue 2, pp. 1–23). MDPI AG. https://doi.org/10.3390/nano11020496

Yong, X., & Du, K. (2023). Effects of Shape on Interaction Dynamics of Tetrahedral Nanoplastics and the Cell Membrane. *Journal of Physical Chemistry B*, *127*(7), 1652–1663. https://doi.org/10.1021/acs.jpcb.2c07460

Yuan, A. Q., Yang, I., Yuan, R. Q., Zhao, L., Bai1, L., & Meng, Q. (n.d.). *Targeting CD19 for the treatment of B-cell related autoimmune diseases with a novel T cell engager*. https://doi.org/10.1101/2024.02.17.580750

Zhang, K., Hamidian, A. H., Tubić, A., Zhang, Y., Fang, J. K. H., Wu, C., & Lam, P. K. S. (2021). Understanding plastic degradation and microplastic formation in the environment: A review. *Environmental Pollution*, *274*, 116554. https://doi.org/https://doi.org/10.1016/j.envpol.2021.116554

Zhang, Y.-X., Wang, M., Yang, L., Pan, K., & Miao, A.-J. (2022). Bioaccumulation of differently-sized polystyrene nanoplastics by human lung and intestine cells. *Journal of Hazardous Materials*, *439*, 129585. https://doi.org/https://doi.org/10.1016/j.jhazmat.2022.129585

REFERENCES

7. ANNEXES

7.Annexes

7.1. The pdf of the published paper entitled Hazard assessment of different-sized polystyrene nanoplastics in hematopoietic human cell lines

ANNEXES

Chemosphere 325 (2023) 138360

Contents lists available at ScienceDirect

Chemosphere

Hazard assessment of different-sized polystyrene nanoplastics in hematopoietic human cell lines

Alireza Tavakolpournegari [a,1], Balasubramanyam Annangi [a,1], Aliro Villacorta [a,b], Gooya Banaei [a], Joan Martin [a], Susana Pastor [a], Ricard Marcos [a,*,1], Alba Hernández [a,1,**]

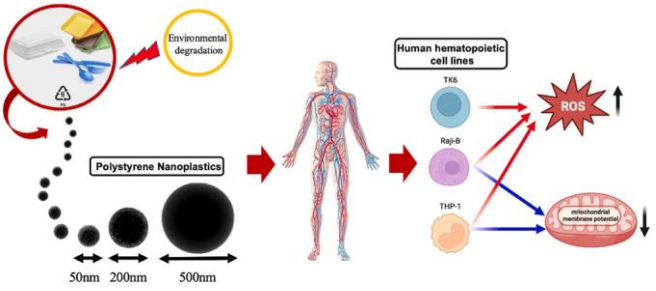
[a] Group of Mutagenesis, Department of Genetics and Microbiology, Faculty of Biosciences, Universitat Auto`noma de Barcelona, Cerdanyola Del Vall`es, Barcelona, Spain

[b] Facultad de Recursos Naturales Renovables, Universidad Arturo Prat, Iquique, Chile

HIGHLIGHTS

- Polystyrene nanoplastics were evaluated in Raji-B, THP-1, and TK6 cells.
- TEM, confocal microscopy, and flow cytometry demonstrated cell internalization.
- Cell uptake was negatively associated with the size.
- Mitochondrial membrane potential loss was induced in Raji-B and THP-1 cells.
- Size, biological endpoint, and cell type modulate the toxicological profile of MNPLs.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Michael Bank

Keywords:

Polystyrene nanoplastic size
Raji-B/THP-1/TK6 cells
Uptake

Oxidative stress

Mitochondrial membrane potential

ABSTRACT

The environmental presence of micro/nanoplastics (MNPLs) is an environmental and human health concern. Such MNPLs can result from the physicochemical/biological degradation of plastic goods (secondary MNPLs) or can result from industrial production at that size, for different commercial purposes (primary MNPLs). Inde- pendently of their origin, the toxicological profile of MNPLs can be modulated by their size, as well as by the ability of cells/organisms to internalize them. To get more information on these topics we have determined the ability of three different sizes of polystyrene MNPLs (50, 200, and 500 nm) to produce different biological effects in three different human hematopoietic cell lines (Raji-B, THP-1, and TK6). Results show that none of the three sizes was able to induce toxicity (growth ability) in any of the tested cell types. Although transmission electron microscopy and confocal images showed cell internalization in all the cases, their quantification by flow cytometry demonstrated an important uptake by Raji-B and THP-1 cells, in comparison with TK6 cells. For the first ones, the uptake was negatively associated with the size. Interestingly, when the loss of mitochondrial membrane potential was determined, dose-related effects were observed for Raji-B and THP-1 cells, but not for TK6 cells. These effects were observed for the three different sizes. Finally, when oxidative stress induction was evaluated, no clear effects were observed for the different tested combinations. Our conclusion is that size, biological endpoint, and cell type are aspects modulating the toxicological profile of MNPLs.

* Corresponding author. Group of Mutagenesis, Department of Genetics and Microbiology, Faculty of Biosciences, Universitat Auto`noma de Barcelona, Campus of Bellaterra, 08193, Cerdanyola Del Vall`es, Spain.

** Corresponding author.
E-mail addresses: ricard.marcos@uab.cat (R. Marcos), alba.hernandez@uab.cat (A. Herna´ndez).
[1] Both authors contribute equally.

https://doi.org/10.1016/j.chemosphere.2023.138360
Received 14 January 2023; Received in revised form 25 February 2023; Accepted 8 March 2023
Available online 10 March 20230045-6535/

ANNEXES

1. Introduction

Environmental plastic waste constitutes a serious ecological problem since they degrade slowly and remain in the environment for hundreds of years (Kik et al., 2020). In addition, these environmental plastics further degrade into micro/nanoplastics (MNPLs). Fragmented or otherwise miniaturized plastic materials in the form of MNPLs are of nagging environmental concern. Since the ubiquitous presence of MNPLs in the environment is inevitable, they can be internalized by any kind of organism, including humans. Thus, the assessment of MNPLs as potential health risk factors for humans demands an urgent response (Yong et al., 2020; Rubio et al., 2020a; Mamun et al., 2023).

Environmental plastic waste differs in its chemical composition. Approximately 90% of the total amount of plastics consists of high- density polyethylene, low-density polyethylene, polyvinyl chloride, polystyrene, polypropylene, and polyethylene terephthalate (Tursi et al., 2022). Consequently, it is assumed that environmental pollutants MNPLs are representative of these proportions. One of the main plastics used for packaging, commercial, and construction purposes is poly- styrene (PS) (Kik et al., 2020). In addition, pristine PSMNPL beads are also used in the production of cosmetics (Xu et al., 2020). Environmental PSMNPLs, either because of plastic degradation goods (secondary MNPLs) or as produced at that size for different industrial purposes (primary MNPLs), enter the exposed organism inducing a wide range of hazardous effects (Qiao et al., 2022). Ingestion is considered the main way of entry of PSMNPLs into the human body. Using *in vitro* models of the intestinal barriers PSNPLs have shown their ability to translocate the barrier and affect lymphocytic cells placed after the intestinal barrier (Domenech et al., 2020). This supposes that once internalized, PSNPLs can interact with blood cell components (as a first instance), later reaching other organs and tissues. Consequently, hematopoietic cells are considered a target of PSNPLs exposure.

The potential toxic effects of PSNPLs may change by various factors, including size and surface charge. In general, it is assumed that small size and positive charge favor cell internalization and hazardous effects (Halimu et al., 2022). Nevertheless, the effect of surface charge is ambiguous, with negatively charged PSNPLs inducing a higher agglomeration modulating the toxic effects in ascidians (Eliso et al., 2020). Furthermore, positively charged PSMNPLs were more toxic on human nasal epithelial cells than negatively charged ones (Huang et al., 2022). Regarding the size effect, it is assumed that smaller sizes can easily be internalized by cells and cross biological barriers (Ramsperger et al., 2022). Nevertheless, *in vivo* results are contradictory. Thus, in the white-leg shrimp, although small PS sizes were more bioavailable and exhibited greater damage in the guts and gills, larger sizes increased the biomarkers of oxidative stress and altered microbiota components (Zhou et al., 2023). In zebrafish, larvae exposed to high-density polyethylene showed that small size caused morphological changes in the gastroin- testinal cells while larger size damaged the mechanosensory receptors in the fish's lateral line system (Kim et al., 2022). Especially relevant is the study carried out in mice with two different-sized polyethylene MPLs testing different parameters detecting intestinal function (gene expres- sion related to epithelial, permeability, and inflammatory biomarkers) where the most marked deleterious effects were found after co-exposure with the two sizes (Djouina et al., 2022).

Aiming to determine the role of MNPL size on their potentially hazardous effects, we have assessed the toxic effects of different sizes of PSNPLs (50, 200, and 500 nm). Since the blood compartment is considered the first target, once MNPLs have crossed the biological barriers, blood cells were selected. Thus, three different human he- matopoietic cell types such as Raji-B (as a B-lymphocyte model), THP-1 (as a macrophage model), and TK6 (as a lymphoblastoid model) were used. Cell viability, cellular uptake, intracellular ROS generation, and mitochondrial dysfunction were the evaluated effects.

2. Materials and methods

2.1. Characterization of the used PSNPLs

Three different sizes of pristine polystyrene nanoplastics were pur- chased from Spherotech Inc. (Chicago, USA). Namely, PSNPLs 50 nm (PS-50; PP-008-10; with nominal sizes of 0.05–0.1 μM), PSNPLs 200 nm (PS-200; PP-015-10; with nominal sizes of 0.1–0.2 μM), and PSNPLs 500 nm (PS-500; PP-05-10; with nominal sizes of 0.4–0.6 μM. All used PSNPLs were supplied as water dispersions.

For their characterization, PSNPLs dispersions were diluted to the concentration of 100 μg/mL in distilled water, or in culture medium (supplemented RPMI-1640, Biowest Inc.) supplemented with 10% fetal bovine serum (FBS), 1% glutamine, 2.5 μg/mL Plasmocin, and then analyzed by transmission electron microscopy (TEM) with a JEOL JEM-1400 instrument (JEOL LTD, Tokyo, Japan). In addition, the hydrody- namic size and the Z-potential parameters were assessed by dynamic light scattering (DLS) and laser Doppler velocimetry (LDV) methodolo- gies, using a Malvern Zetasizer Nano ZS zen3600 device (Malvern, UK).

2.2. Labeling and detection of PSNPLs

To visualize PS-50, PS-200, and PS-500 by confocal microscopy, the particles were labeled with the commercially available textile dye *iDye Poly Pink*, from now iDye. The procedure was adapted from previously published protocols for labeling micro and nanoscale polymers (Karak- olis et al., 2019; Nguyen and Tufenkji, 2022). Briefly, the steps were performed as follows. Suspensions of 1 mL of de different PSNPLs, at a final concentration of 5 mg/mL were prepared and transferred to 1.5 mL tubes, containing 0.01 g of iDye. The mixture was vigorously vortexed
and transferred to a 10 mL glass tube and incubated for 2 h at 70 ˚C, and then cooled at room temperature. Nine mL of Milli-Q water was then added to the tube, and the suspension was centrifuged at 4000 rcf on an Amicon® Ultra-15 centrifugal Ultracel®-100 K filter 1 × 10 5 MWCO for
15 min. This step was repeated twice to remove the excess of iDye. The stained particles were stored at 4 ˚C protected from light until needed. To determine the labeling and fluorescence spectra, particles were examined by confocal microscopy. To such end, working suspensions at the concentration of 400 μg/mL were prepared. Two drops (20 μL) were placed on previously ethanol-washed glass slides and covered with a thin glass coverslip. The whole procedure was carried out under a laminar flow cabinet. Aggregates of particles were then analyzed using a Leica TCS SP5 confocal microscope. The excitation wavelength was set at 561 nm, and the emission spectra were collected between 585 and 700 nm. The emission was analyzed using the Leica Application Suite X 3.7.5.24914 (Leica Microsystems CMS GmbH Wetzlar, Germany). Im- ages were collected under the same conditions, as further explained in section 2.9.

2.3. Cell culture

Human hematopoietic cell lines TK6, THP-1, and Raji-B were used for this study. All the cells were purchased from Sigma Aldrich (MO, USA). TK6 as a human lymphoblastoid cell line is one of the standard mammalian cell lines used for *in-vitro* genotoxicity/mutagenicity tests. THP-1 is a human leukemia monocytic cell line, which is extensively used to study monocyte/macrophage functions, toxicity mechanisms, signal-transduction pathways, and nutrient and drug transport. Furthermore, Raji-B is the first continuous human B lymphocyte cell line of hematopoietic origin. All the mentioned cell lines were maintained in supplemented RPMI-1640 (Biowest Inc) medium supplemented with 10% FBS, 1% glutamine, and 2.5 μg/mL Plasmocin. All cell lines were
cultured at 37 ˚C and 5% CO_2 and, to maintain the cells, the medium was changed with fresh medium every 2–3 days.

ANNEXES

2.4. Treatment of cells

To study the potentially toxic effects of PSNPLs, cells were exposed to the selected concentrations of PSNPLs (PS-50, PS-200, and PS-500) diluted in RMPI 1640 supplemented medium, as described in the previous section. To proceed, the density of 5×10^5 cells/mL in the volume of 200 µL were seeded on 96 well plates (1×10^5 cells/well) and exposed to the different concentrations of PSNPLs and grown for 3 h, 24 h, and 48 h, according to the experimental endpoints.

2.5. Cell viability assay

The viability of the three cell lines after exposure was measured using the Beckman counter method. After seeding cells on 96 well plates with a concentration of 5×10^5 cells/mL, they were exposed to the different concentrations of PSNPLs (0, 50, 100, 150, and 200 µg/mL) for 24 h to assess cell toxicity. After exposure, cells were diluted 1:100 to ISOTON and counted with a ZTM Series Coulter counter (Beckman Coulter Inc., CA). The obtained percentage values were calculated as the average number of the cells counted for each treatment, compared to the untreated control culture values.

2.6. Cellular uptake assessment with transmission electron microscopy (TEM)

Among the different tools to assess PSNPLs internalization, TEM was used. To such end, cells were grown in T25 cell culture flasks to reach the density of 1.7×10^6 cells/mL. Then, each flask was treated with 3 mL of a concentration of 50 µg/mL of each PSNPL, previously diluted in RMPI 1640, for 24 h. After exposure, cells were centrifuged and the pellet was fixed in 2.5% (v/v) glutaraldehyde (EM grade, Merck, Darmstadt, Germany) and 2% (w/v) paraformaldehyde (Hatfield, PA, UK) in 0.1 M cacodylate buffer (Sigma-Aldrich, Steinheim, Germany), pH 7.4. Cells were processed following conventional procedures, as previously described (Annangi et al., 2015). Briefly, samples were first post-fixed with osmium trioxide, dehydrated in acetone, later embedded in Eponate 12™ resin (Ted Pella Inc., Redding, CA), and finally polymerized at 60 °C and cut with an ultramicrotome. Ultrathin sections were placed in copper grids and contrasted with uranyl acetate and Reynolds lead citrate solutions and observed using a JEOL 1400 (JEOL LTD, Tokyo, Japan) transmission electron microscope equipped with an Erlangshen (CCD GATAN ES1000W) camera.

2.7. Cellular uptake and localization of PSNPLs analysis with confocal microscopy

The iDye-labeled PSNPLs (iDye-PSNPLs) were used to determine PSNPLs' internalization by confocal microscopy in the three selected cell lines. To this end, 80,000 cells were seeded in glass bottom microwell dishes (MatTek, Ashland, OR, USA) and exposed to iDye-PSNPLs (100 µg/mL) for 24 h. The samples were washed with PBS, and nuclei and cell membranes were stained with 1:500 Hoechst 33,342 (ThermoFisher Scientific, Carlsbad, CA, USA) and 1:500 Cellmask™ Deep Red plasma (ThermoFisher Scientific, Carlsbad, CA, USA) respectively, for 15 min at room temperature. The iDye-PSNPLs of different sizes were visualized intracellularly at emission wavelengths of 585 nm, under a Leica TCS SP5 confocal microscope. Several fields were selected randomly per sample, and the images were processed using ImageJ software having Fiji extension.

2.8. PSNPLs internalization assessment with flow cytometry

Aiming to quantify the cellular uptake of PSNPLs, flow cytometry was assessed. All three cell lines were treated for 24 h with 100 µg/mL of labeled iDye-PSNPLs. After exposure, cells were washed, collected, centrifuged, and resuspended to 7.5×10^5 cells/mL in PBS. The fluorescence inside the cells was measured by flow cytometry (CytoFlex, Beckman Coulter, USA) with excitation/emission spectra of 561/630 nm respectively. A total number of 10,000 cells were scored for all the conditions, and the data were analyzed using the Cytexpert software.

2.9. Intracellular ROS production and analysis

The production of intracellular ROS was measured by the dihydroethidium (DHE) method. The three cell lines were exposed to PSNPLs with different concentrations ranging from 0 to 50 µg/mL for 3, 24, and 48 h. After the end of the exposure time, the cells were centrifuged, pelleted, and incubated with 10 µM of DHE in PBS for 30 min at 37 °C. After DHE exposure, cells were analyzed by flow cytometry (CytoFlex, Beckman Coulter, USA). As a positive control, cells were treated with 80 µM of antimycin-A and incubated for 45 min at 37 °C. The fluorescent intensity was measured at 488–605 nm (excitation-emission spectrum) and 10,000 cells per sample were scored and analyzed with the Cytexpert software.

2.10. Mitochondrial membrane potential assay

To determine potential mitochondrial damage caused by PSNPLs in Raji-B, THP-1, and TK6 cells, the mitochondrial membrane potential assay was utilized (MitoProb TMRM assay kit, Thermofisher Scientific, USA). The cells were seeded in 96 well plates with a concentration of 5×10^5 cells/mL and exposed to all sizes of PSNPLs with concentrations (0, 50, and 100 µg/mL) lasting for 24 h. After exposure, the cells were washed twice with PBS and then they were centrifuged and the pellet obtained was resuspended in PBS. The fluorescence intensity was measured by flow cytometry (CytoFlex, Beckman Coulter, USA) with the excitation/emission of 561/585 nm, respectively. For each concentration, the total number of 10.000 cells was scored and data was analyzed by the Cytexpert software.

2.11. Statistical analysis

All data resulted from the average of three independent experiments, including duplicates of each one of them. Data were analyzed using GraphPad Prism 7 software, and the statistical analysis was performed using the two-way ANOVA with Tukey's multiple comparison test unless stated otherwise. Statistical significance was defined as a $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

3. Results and discussion

3.1. Characterization and dispersion of PSNPLs

The present study determined the average size and shape of the supplied pristine PSNPLs, with nominal sizes of 100–200, 400–600, and 400–900 nm by using TEM. The obtained average sizes for PS-50, PS-200, and PS-500 wetted in distilled H_2O, or in supplemented RPMI-1640 medium, are indicated in Fig. 1. In general, they are in accordance with the manufactured specified range of sizes. Furthermore, all the shapes of the used PSNPLs were spherical, both in distilled H_2O and in supplemented RPMI-1640. Table 1 shows the hydrodynamic radius (or mean size diameter) determined with a Zetasizer Nano ZS device, both in distilled H_2O and in supplemented RPMI-1640. It is important to point out the protein corona generated when dispersed in the culture medium, and the tendency to agglomerate when dispersed in supplemented RPMI-1640 over water dispersion. Moreover, the measurement of the Z-potential in supplemented RPMI-1640 revealed less stability, as reflected by the lower Zeta values when compared to PSNPLs dispersed in H_2O. Finally, the polydispersity index (PDI) values were close to zero suggesting they were monodispersed.

ANNEXES

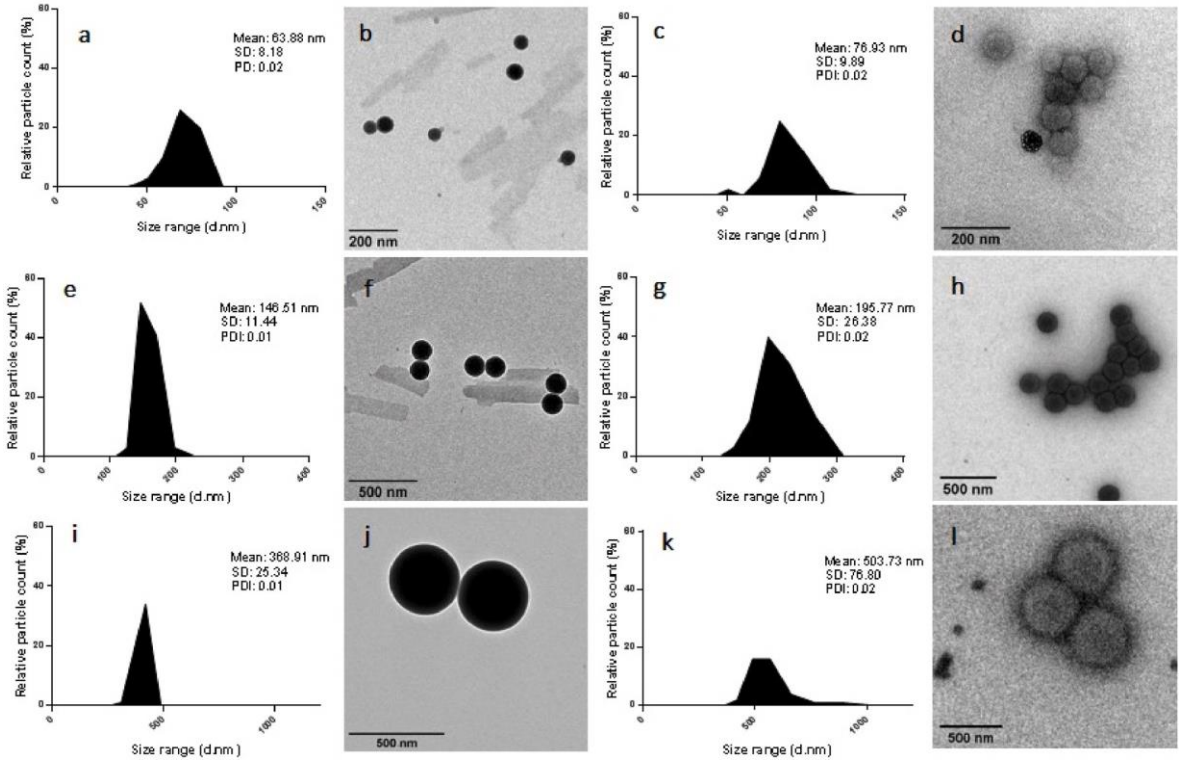


Fig. 1. The graphs indicate the size distribution (mean ± SD) and polydispersity index in water and in supplemented RPMI-1640, of PS-50 (a, c); PS-200 (e, g); and PS-500 (i, k). The TEM images show round shapes in both water and in supplemented RPMI-1640 for PS-50 (b, d), PS-200 (f, h), and PS-500 (j, l).

Table 1

Characteristics of the selected PSNPLs when dispersed in water or in supplemented RPMI-1640 cell culture medium. The hydrodynamic radius or average size in diameter (Z-average), the zeta potential, and the polydispersity index (PDI) of PS-50, PS-200, and PS-500 nm are shown, Data are represented as (mean ± SD).

	Milli-Q H_2O			SUPPLEMENTED RPMI-1640		
	PS-50	PS-200	PS-500	PS-50	PS-200	PS-500
Z-average	94.00 ± 0.86	188.00 ± 2.98	488.00 ± 7.09	94.50 ± 0.39	185.00 ± 2.10	588.70 ± 5.37
PDI	0.03 ± 0.02	0.04 ± 0.03	0.03 ± 0.02	0.09 ± 0.01	0.05 ± 0.03	0.26 ± 0.13
Z-potential	—44.90 ± 0.49	—45.00 ± 0.24	—50.40 ± 0.94	—11.70 ± 3.05	—10.10 ± 1.86	—16.00 ± 0.81

3.2. Visualization and spectral analysis

PSNPLs were successfully labeled with iDye, using the previously reported method (Nguyen and Tufenkji, 2022). The colored particles were easily visualized by confocal microscopy in all three cases (Fig. 2a and b, c) and the obtained emission spectra from 585 to 700 nm are depicted at the right of each image. The peak of emission was consis- tently between 610 and 620 nm. On the other hand, no fluorescence was detectable for any of the not labeled PSNPLs. Interestingly, no back- ground fluorescence was observed indicating a lack of leaching.

3.3. Cell viability

To assess the cytotoxic potential of the three different PSNPLs' sizes, TK6, THP-1, and Raji-B cells were exposed to a range of concentrations (0–200 μg/mL). The obtained results are indicated in Fig. 3. The data revealed no significant decrease in cell viability, independently of the PSNPLs size and the concentration used, after exposures lasting for 24 h. Since none of the concentrations of PSNPLs in any of the cell lines was cytotoxic, the study further utilized and tested the sub-toxic doses of PSNPLs (≤100 μg/mL) for the rest of the experiments. In agreement, the exposure of PS-50 to the same cell types (Raji-B, THP-1, and TK6) as well as in human colorectal adenocarcinoma (Caco-2) cells did not result in a

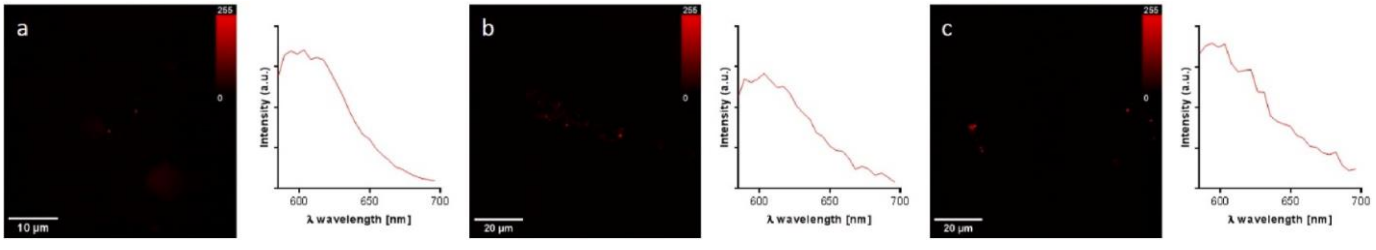


Fig. 2. Visualization of PS-50, PS-200, and PS-500 labeled with iDye Poly Pink (a, b, and c, respectively). At the right, their respective spectra are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

ANNEXES

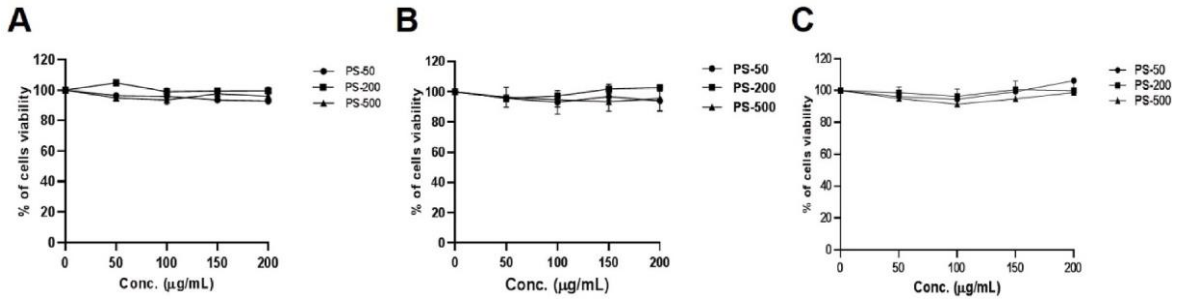


Fig. 3. Cell viability effects of different sizes and concentrations of PSNPLs in various hematopoietic cell lines. (A) The percentage of cell viability in Raji-B cells. (B) The percentage of cell viability in THP-1 cells. (C) The percentage of cell viability in TK6 cells. Exposures last for 24 h. Data are represented as (mean ± SEM).

decrease in cell viability in any of them and at various concentrations and time points (Cort´es et al., 2020; Rubio et al., 2020b). Besides, PSNPLs (100 nm and 1 µm) exposure in mouse hippocampal neuronal (HT22) cells yielded a mild cytotoxic response only with the smaller sized PS-100 but not PSNPLs of 1 µm indicating a possible size effect (Liu et. al 2022). In addition, PSNPLs (20 and 70 nm) were able to reduce cell viability when used to expose human alveolar epithelial cells (A549) at different concentrations (Xu et al., 2019).

3.4. Cellular internalization of PSNPLs using different analytical tools

The assessment of cellular internalization or uptake of PSNPLs was undertaken by utilizing various analytical tools like TEM, confocal microscopy, and flow cytometry considering they could complement one another and overcome the limitations among them to provide robust information on the cellular internalization of PSNPLs.

3.5. Transmission electron microscopy (TEM)

The first approach to evaluate the cellular uptake of non-fluorescent pristine PSNPLs in the selected cell types was TEM. This technique helps to locate and visualize PSNPLs in the cell ultrastructure. The obtained TEM images demonstrated the significant internalization of the different sizes of PSNPLs (50 µg/mL, 24 h) in all the selected cell lines (Fig. 4). According to the observed micrographs, PSNPLs were found localized in or near different cellular compartments based on their size, apart from being present in the cytoplasm of the cells. TEM images showed PSNPLs incorporation in different cell organelles like mitochondria of Raji-B

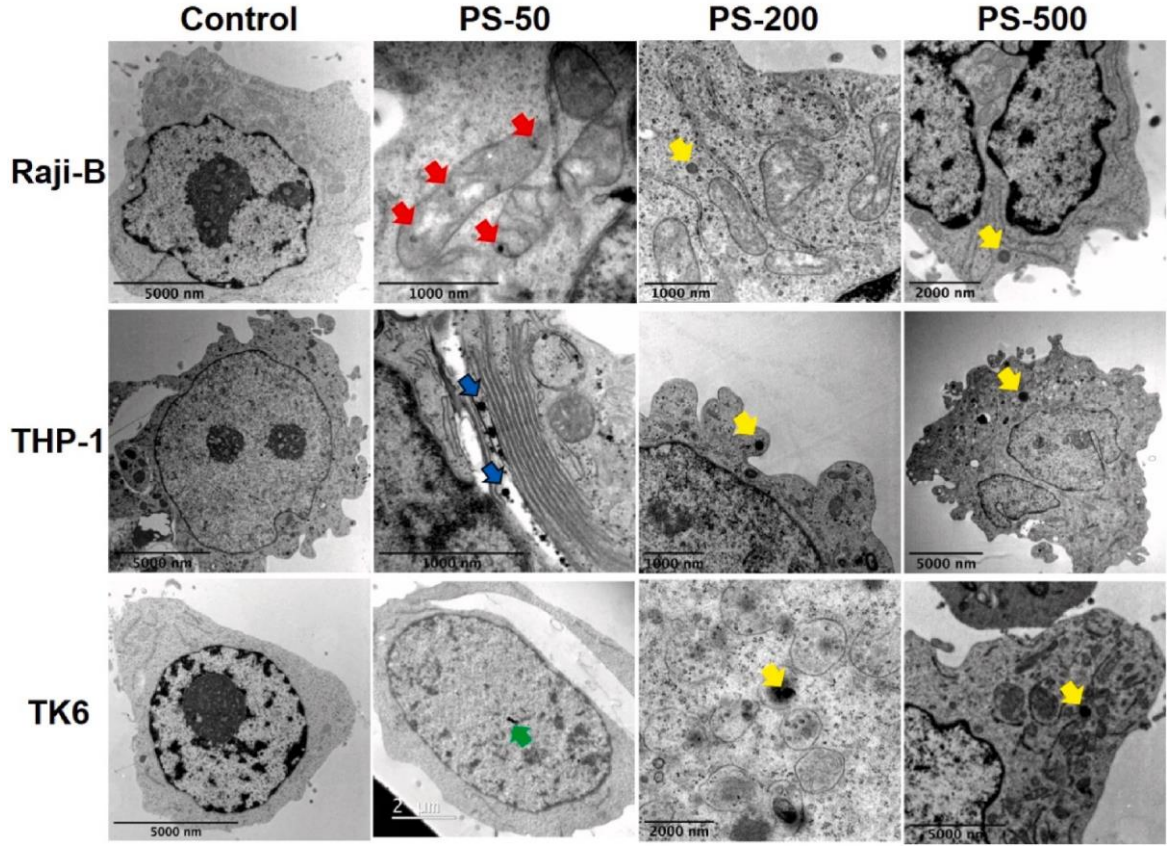


Fig. 4. Representative TEM images showing the cellular internalization and localization of the three different sizes of pristine PSNPLs in Raji-B, THP-1, and TK6 cells after treatments lasting for 24 h. Arrows in red indicate PSNPLs in mitochondria, yellow in cytoplasmic regions, green in the nucleus, and blue in the endoplasmic reticulum. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cells and endoplasmic reticulum of THP-1 cells. Furthermore, they were able to reach the nuclei, as observed in TK6 cells.

3.5.1. Confocal microscopy

Due to the success in staining PSNPLs (iDye-PSNPLs) they could be used in confocal microscopy. The confocal images of the treated cells revealed the significant internalization of PS-50, PS-200, and PS-500. They were mostly present in the cytoplasm and surrounding the nuclei in TK6, THP-1, and Raji-B cells after exposures lasting for 24 h (Fig. 5). Moreover, there was a size-dependent cellular internalization in all the cell lines, PS-50 being the most internalized. We assume that this elevated entry of PS-50 was due to their smaller size.

3.6. Flow cytometry

Aiming to quantify the cellular internalization of the different sizes of labeled PSNPLs flow cytometry was used. The intracellular presence of iDye-PSNPLs (100 μg/mL) in the treated cells was determined ac- cording to the fluorescence intensity, as a measure of the percentage of internalized cells. The obtained results are indicated in Fig. 6. Results indicate a cell-dependent internalization capacity according to the ranking Raji-B > THP-1 > TK6. For Raji-B and THP-1 cells, PS-50 were those showing the highest internalization, which would agree with their small size. TK6 cells showed the lowest internalization, although the detected uptake was statistically significant. This data confirms two things, such as a) cell internalization levels depend on the cell type, and b) the uptake is size dependent, showing greater internalization with the smaller size.

In the current study, the data revealed there was a size-dependent cellular internalization, PS-50 being highly internalized over PS-200 and PS-500 when studied in TEM, confocal microscopy, and flow cytometry. Moreover, we found there was a cell type-specific cellular internalization considering Raji-B was more prone to the uptake of PSNPLs followed by THP-1, and the lowest internalization by TK6 cells. In support, the cellular internalization of PS-50 was also observed in the same cell types (Raji-B, THP-1, and TK6) studied by flow cytometry (Rubio et al., 2020b). In agreement with our study, there was a size-dependent cellular internalization of PSNPLs in Caco-2 and A549 cell lines wherein PS-70 were highly internalized as compared to bigger sizes (PS-200 and PS-500), analyzed by both confocal microscopy and flow cytometry, suggesting the smaller sizes were readily accessible by the A549 and Caco-2 cells (Zhang et al., 2022). In addition, Banerjee et al. (2022) indicated that the smaller size of PSNPLs (i.e., 50 nm) was significantly internalized in HepG2 cells as compared to PS-200 and PS-500 nm, when visualized by confocal microscopy. Furthermore, it may be pointed out that the smaller size tested (i.e., PS-70 nm) would enter *via* clathrin- and caveolae-mediumted endocytosis as well as phagocytosis, while the larger particles like PS-200 and PS-500 nm could enter the A549 and Caco-2 cells only through phagocytosis and this could explain the higher internalization of the smallest NPLs (Zhang et al., 2022).

The usage of different analytical tools for cellular internalization in the present study was justified as no one tool could completely capture the NPL's internalization thoroughly due to various factors. For instance, the low atomic number of polymers in MNPLs could render them to scatter electrons weakly resulting in poor contrast under TEM (Sawyer et al., 2008). In addition, it warrants laborious sample prepa- ration and time consumption which possibly make it irrelevant to analyze the high number of particles needed for representative studies (Ivleva, 2021). Moreover, MNPLs labeled with fluorophores could

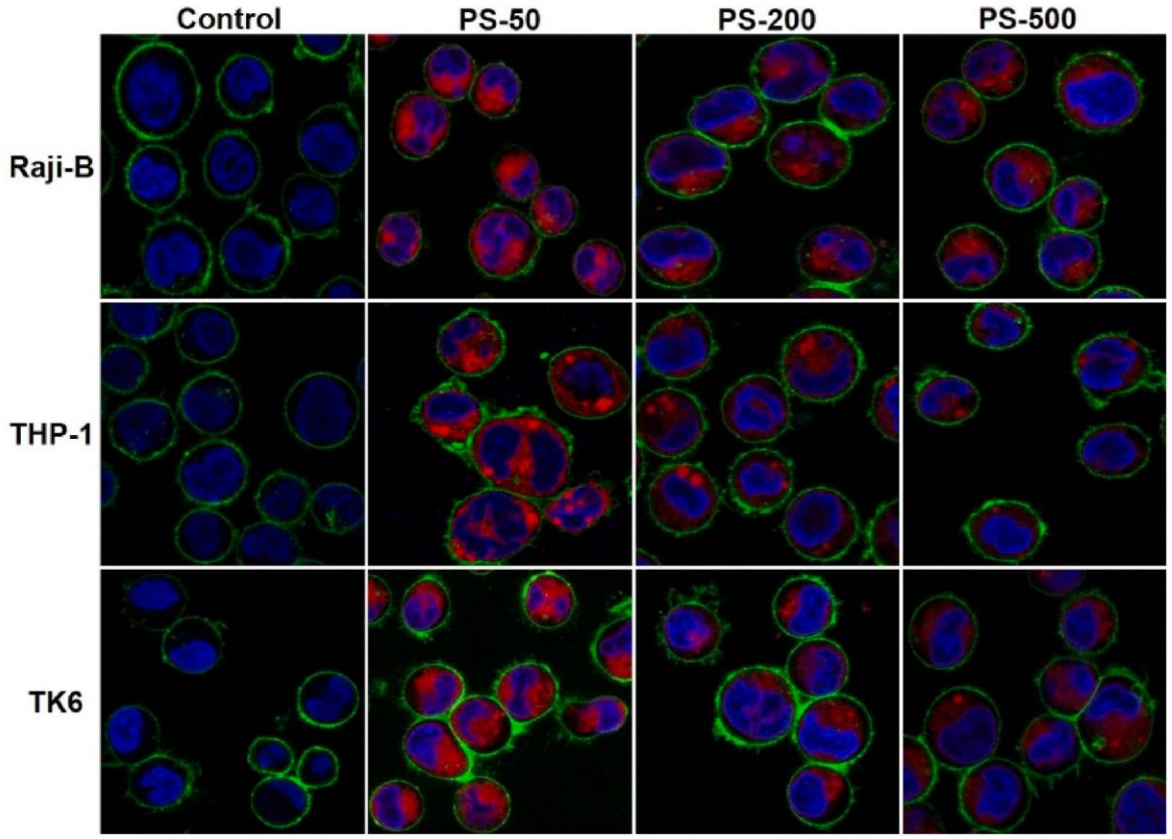


Fig. 5. Confocal microscopy images showing the cellular internalization of different sizes of iDye-PSNPLs (100 μg/mL) in Raji-B, THP-1, and TK6 cells after treatments lasting 24 h. Fluorescent iDye labeled PSNPLs are red, the cell membranes are green (cell mask), and the nuclei of the cells are blue (Hoechst 33,342). Magnification 63x. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

ANNEXES

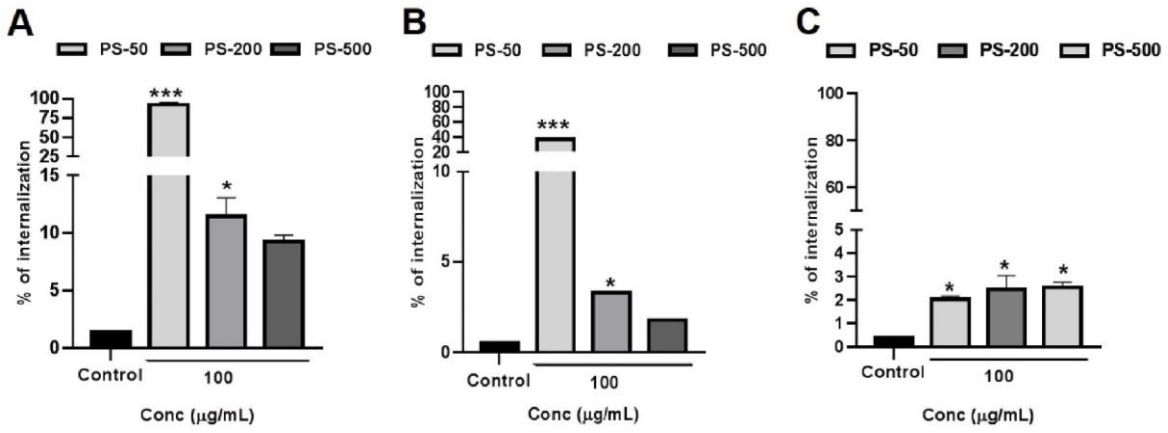


Fig. 6. Histograms showing the cell internalization of the different sizes of PSNPLs in the selected hematopoietic cells by using flow cytometry. (A) The percentage of cellular internalization in Raji-B cells, (B) in THP-1 cells, and (C) in TK6 cells. Exposures last for 24 h. Data are represented as (mean ± SEM). **P* < 0.05, ****P* < 0.001 (2-way ANOVA and Student's *t*-test).

possibly shed the fluorophores in cell culture medium thereby the observation of fluorescence intracellularly might be due to free fluo- rophores (Tenuta et al., 2011). Also, the potential leaching effect of fluorophores from the labeled PSNPLs was reported (Catarino et al., 2019). Fluorescent-labeled PSNPLs (500–1000 nm) were not able to cross the epithelial barrier of zebrafish larvae rather fluorophores alone were detected in the internal tissues. Apart, the internalization of PSNPLs could be read in flow cytometry due to an increase in the cell complexity; however, the tendency of PSNPLs to adhere to the cell surface may also reflect the increase in cell volume thereby affecting the actual estimation of their internalization in that technique (Lesniak et al., 2013; Zhang et al., 2022).

3.7. Loss of mitochondrial membrane potential

Considering the previous data showing PSNPLs' interaction with mitochondria, potential damage to these organelles was evaluated. Thus, the loss of mitochondrial membrane potential (MMP) was chosen as a biomarker of effect. Flow cytometry was used to assess the loss of MMP, using the TMRM assay, showing that entering of PSNPLs into the cells induced significant effects on the mitochondrial function, by decreasing the MMP values. Results show that all PSNPLs sizes were able to cause the loss or decrease in MMP values at the tested concentrations (50 and 100 µg/mL) in Raji-B and THP-1, cells when compared to their respective controls after 24 h ($P < 0.05$) (Fig. 7). On the other hand, TK6 cells did not show any change in MMP levels at the tested concentra- tions, as compared to their corresponding untreated controls after exposure to all sizes of PSNPLs for 24 h. This lack of effect could possibly be attributed to the non-interaction of PSNPLs with the mitochondria of the TK6 cells, as it was observed by TEM, where no presence of PSNPLs in or near the mitochondria of TK6 cells could be detected. Like our data on the size-dependent loss of MMP in Raji-B cells, a previous study also demonstrated the size-dependent MMP effects due to exposure of Caco- 2 cells to PSMPLs sized 0.1 and 5 µm. The cells treated with 5 µm PSMPLs had a greater loss of MMP as compared to 0.1 µm sized PS (Wu et al., 2019). On the other hand, the smallest size of PSNPLs had a sig- nificant effect on the mitochondrial membrane potential in THP-1, possibly indicating a cell type-specific effect. Besides, there is no specific study comparing the size effect of PSNPLs on the mitochondrial dysfunction in THP-1 cells, our data proved relevant since the exposed THP-1 cells were more responsive to different sizes of PSNPLs, in com- parison to the assessed cells like TK6. Moreover, amino-modified PSNPLs (50 nm) showed a profound effect on mitochondrial metabolic activity and in the loss of MMP in a time and dose-dependent manner, as observed in the exposed alveolar macrophages (Deville et al., 2020). Similarly, amino-modified PS-20, unmodified PS-20, and PS-50 nm caused loss of MMP, enhanced proton leak from mitochondria, as well as elevated adenosine triphosphate (ATP) generation, and mitochondrial oxidative phosphorylation in a dose-dependent way in A549 cells (Halimu et al., 2022).

3.8. Induction of intracellular reactive oxygen species (iROS)

To understand the adverse health effects of PSNPLs from a biological point of view, different biomarkers could be analyzed in human cells. One of the most important biomarkers detecting cell toxicity and cellular stress could be iROS. The role of nascent oxygen species in cell signaling under normal conditions was well established; however, their imbalance due to oxidative stress could trigger DNA damage or cellular death (Bidooki et al., 2022). Furthermore, the imbalance in the oxygen free radicals could be implicated in many debilitating diseases such as cancer-causing, cardiovascular, and neurological disorders, among others (Frijhoff et al., 2015). The assessment of the generation of iROS, by PSNPLs exposure in the different cell types, showed that there were significant size- and time-dependent increases in the percentage of iROS in all the tested cell lines. Interestingly, significantly elevated levels of iROS were found in Raji-B cells treated with larger-sized PSNPLs (e.g., PS-500; 25 µg/mL) compared to PS-50 and PS-200 nm (Fig. 8). On the other hand, PS-200 treated THP-1 (25 µg/mL) and TK6 (5–50 µg/mL) cells showed a significantly increased production of iROS (according to the results shown in Fig. 8), in comparison to PS-50 and PS-500 nm after exposures lasting for 3 h ($P < 0.05$).

Furthermore, when the exposure time was increased to 24 h, resulted in significant increases in iROS in PS-500 treated THP-1 only (10–50 µg/ mL) ($P < 0.05$) (see Supplementary Fig. S1), indicating the possible time and cell-dependent transient effect. In addition, the obtained data did not indicate any increase in iROS with an increase in time up to 48 h for all sizes of PSNPLs and for all the hematopoietic cell types (see Supplementary Fig. S2).

The potential of PSNPLs to induce iROS was reported in several studies as well. Like our study, Rubio et al. (2020b) assessed the gen- eration of iROS in the same cell types (Raji-B, THP-1, and TK6) after treating them with PS-50 at different concentrations and time points. The study demonstrated that there was a cell type-dependent induction of iROS since PS-50 (at 50 µg/mL) treated TK6 and THP-1 cells showed significant increases of iROS after 3 h, albeit there were more elevated in TK6 even after 24 h post-treatment. On the contrary, THP-1 cells were not responsive to PS-50 nm induced iROS at any of the doses and time points. Furthermore, a recent study also showed that PSNPLs could trigger iROS in a size-dependent manner in Caco-2 cells wherein the

ANNEXES

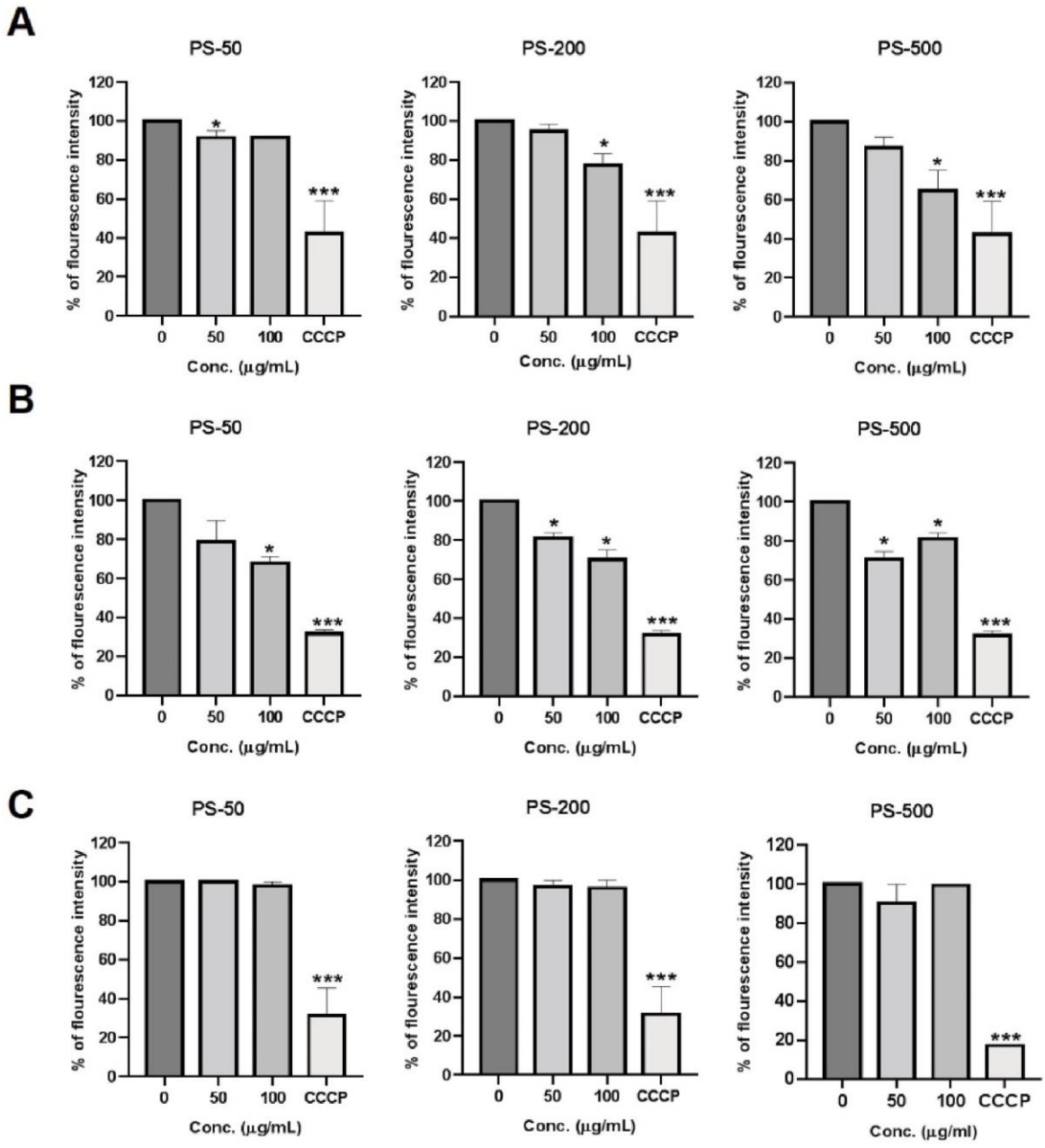


Fig. 7. The graphs indicate the loss of mitochondrial membrane potential after exposure to different sizes of PSNPLs viz. PS-50, PS-200, and PS-500 nm in Raji-B (A), THP-1 (B), and TK6 cells (C) after 24 h of treatments. Data are represented as (mean ± SEM). $*P < 0.05$, $***P < 0.001$ (2-way ANOVA and Student's t-test).

smaller-sized PS-300 were able to induce higher iROS, when compared to PS-500 nm and micron-sized PSNPLs (Wang et al., 2020). Similarly, PSNPLs of 40 nm were able to alter the oxidative status in the exposed human bronchiolar (BEAS-2B) and in alveolar (HPAEpiC) epithelial cells, as there were significantly elevated levels of iROS in them after 24 h, which could possibly be implicated in lung injury (Yang et al., 2021). Moreover, the surface functionalized as well as non-functionalized PSNPLS with varying sizes (amino-functionalized PS-20, non-- functionalized PS-20, and PS-50 nm) triggered a dose-dependent sig- nificant increase in iROS, as well as the role of increased expression of NADPH oxidase 4 in PSNPLs induced epithelial to mesenchymal tran- sition in A549 cells (Halimu et al., 2022). Similarly, the ability of ami- nated PSNPLs to induce ROS has been recently detected in murine alveolar macrophage (MH-S) cells (Wu et al., 2023). Looking for the mechanism of production of ROS and the resulting oxidative stress, and using a proteomic approach, the authors detected that the overexpression of the immunoresponsive gene 1 (*irg1*) protein was involved in the increased generation of ROS (as well of other inflam- matory factors). Interestingly, and as we have observed in our study, the induction of ROS by PSNPLs has been associated with the presence of mitochondrial damage in the mouse macrophages RAW 264.7 (Chen et al., 2023). This agrees with our recent data exposing primary human nasal epithelial cells to PSNPLs, where the induction of ROS and the loss of mitochondrial membrane potential were associated with the modu- lation of the autophagy pathway in the form of the accumulation of LC3-II and p62, as models of autophagy markers (Annangi et al., 2023). This link between increased intracellular ROS and reduced mitochon- drial membrane potential (and its consequent mitochondrial damage) has been extensively reviewed by Shadel and Horvath (2015).

In summary, it was evident that PSNPLs had the potential to induce iROS due to several contributing factors like the nature of cell type, their different sizes, doses, and surface characteristics.

ANNEXES

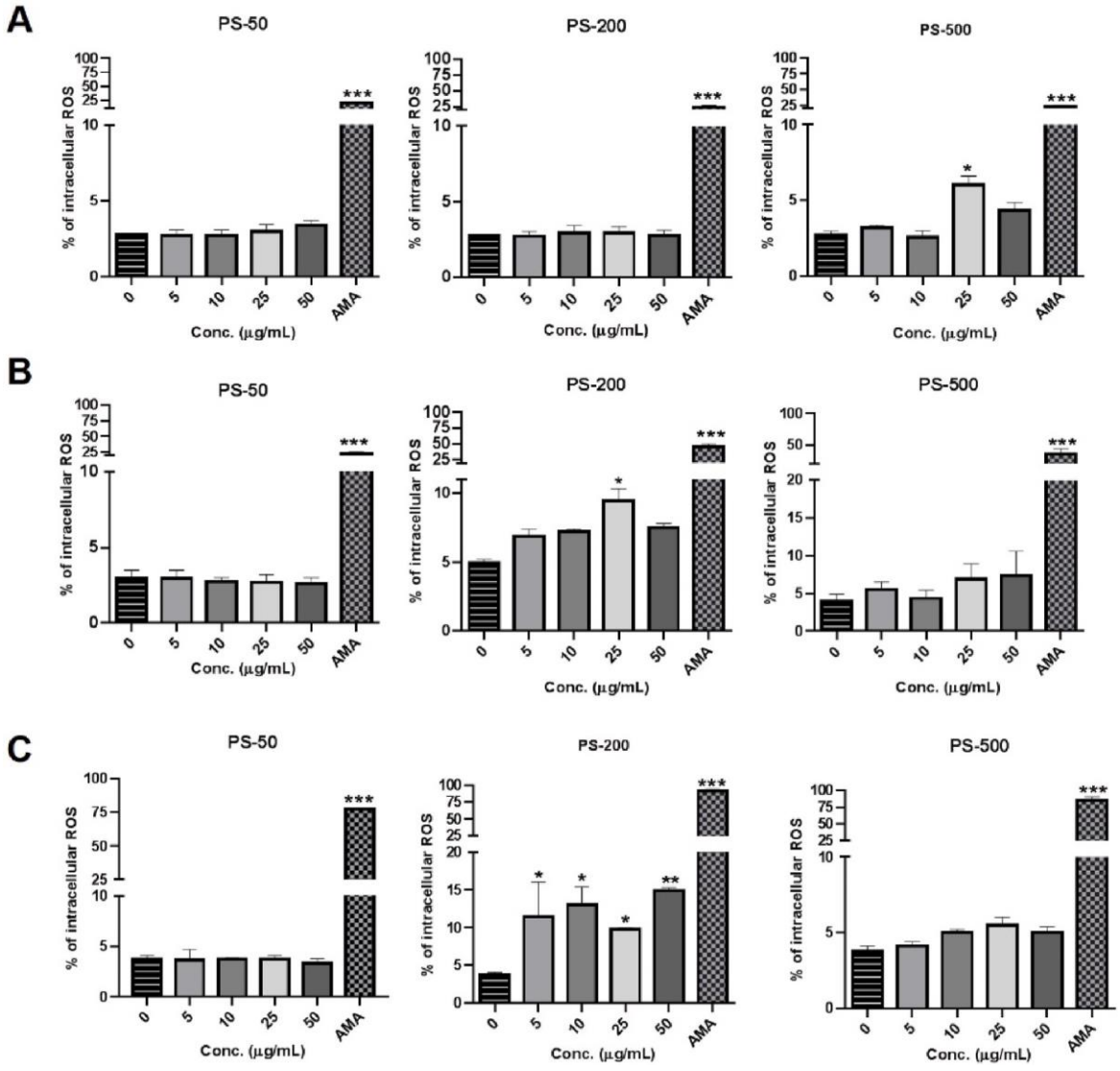


Fig. 8. The histograms show the percentage of intracellular reactive oxygen species due to exposure of different sizes of PSNPLs viz., PS-50, PS-200, and PS-500 in Raji-B (A), THP-1 (B) and TK6 cells (C) after 3 h of treatments. Data are represented as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (2-way ANOVA and Student's *t*-test).

4. Conclusions

The constant weathering or leaching of polymer plastics into sec- ondary MNPLs due to various environmental factors is a major cause of concern for potential human health effects. In context, this study may provide necessary inputs for the hazard and risk assessment of MNPLs like PSNPLs when they encounter important human blood immune cell types through various routes of exposure. The release of MNPLs into the environment in various sizes and forms may need to be considered while assessing their risk. As evidenced, PSNPLs in various sizes could trigger biological responses such as cellular internalization, loss of MMP, and generation of iROS in exposed cells in a time-dependent manner. These biological responses may have cascading effects in altering or modu- lating the cellular functions and survival mechanisms, although they may not necessarily lead to cell death. Moreover, it is also important to understand the chronic toxic effects of PSNPLs with different sizes since there may exist a possibility of constant or continuous environmental exposure to humans.

Author contribution statement

RM and AH planned the experiments. AT, BA, AV, GB, JM, and SP carried out the experimental part. AT and BA analyzed the data, carried out the statistical analysis, prepared tables/figures, and wrote the draft manuscript. BA, RM, and AH wrote the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

AT and JM hold Ph.D. FI fellowships form the Generalitat de Cata- lunya. AV was supported by a Ph.D. fellowship from the National

ANNEXES

Agency for Research and Development (ANID), CONICYT PFCHA/ DOCTORADO BECAS CHILE/2020–72210237. AH was granted an ICREA ACADEMIA award.
This project (Plasticheal) has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 965196. This work was also partially supported by the Spanish Ministry of Science and Innovation [PID 2020-116789, RB-C43] and by the Generalitat de Catalunya (2021-SGR-00731).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.138360.

References

Annangi, B., Bach, J., Vales, G., Rubio, L., Marcos, R., Hern´andez, A., 2015. Long-term exposures to low doses of cobalt nanoparticles induce cell transformation enhanced by oxidative damage. Nanotoxicology 9 (2), 138–147. https://doi.org/10.3109/17435390.2014.900582.

Annangi, B., Villacorta, A., Lo´pez-Mesas, M., Fuentes-Cebrian, V., Marcos, R., Hern´andez, A., 2023. Hazard assessment of polystyrene nanoplastics in primary human nasal epithelial cells, focusing on the autophagic effects. Biomolecules 13, 220. https://doi.org/10.3390/biom13020220.

Banerjee, S., Billey, L.O., McGarvey, A.M., Shelver, W.L., 2022. Effects of polystyrene micro/nanoplastics on liver cells based on particle size, surface functionalization, concentration, and exposure period. Sci. Total Environ. 836, 15562 https://doi.org/ 10.1016/j.scitotenv.2022.155621.

Bidooki, S.H., Alejo, T., Sa´nchez-Marco, J., Martínez-Beamonte, R., Abuobeid, R., Burillo, J.C., Lasheras, R., Sebastian, V., Rodríguez-Yoldi, M.J., Arruebo, M., Osada, J., 2022. Squalene loaded nanoparticles effectively protect hepatic AML12 cell lines against oxidative and endoplasmic reticulum stress in a TXNDC5- dependent way. Antioxidants 11, 581. https://doi.org/10.3390/antiox11030581.

Catarino, A.I., Frutos, A., Henry, T.B., 2019. Use of fluorescent-labelled nanoplastics (NPs) to demonstrate NP absorption is inconclusive without adequate controls. Sci. Total Environ. 670, 915920 https://doi.org/10.1016/j.scitotenv.2019.03.194.

Chen, J., Xu, Z., Liu, Y., Mei, A., Wang, X., Shi, Q., 2023. Cellular absorption of polystyrene nanoplastics with different surface functionalization and the toxicity to RAW264.7 macrophage cells. Ecotoxicol. Environ. Saf. 252, 114574 https://doi.org/ 10.1016/j.ecoenv.2023.114574.

Cort´es, C., Domenech, J., Salazar, M., Pastor, S., Marcos, R., Hern´andez, A., 2020. Nanoplastics as a potential environmental health factor: effects of polystyrene nanoparticles on human intestinal epithelial Caco-2 cells. Environ. Sci.: Nano 7 (1), 272–285. https://doi.org/10.1039/C9EN00523D.

Deville, S., Honrath, B., Tran, Q.T.D., Fejer, G., Lambrichts, I., Nelissen, I., Dolga, A.M., Salvati, A., 2020. Time-resolved characterization of the mechanisms of toxicity induced by silica and amino-modified polystyrene on alveolar-like macrophages.

Arch. Toxicol. 94 (1), 173–186. https://doi.org/10.1007/s00204-019-02604-5. Djouina, M., Vignal, C., Dehaut, A., Caboche, S., Hirt, N., Waxin, C., Himber, C.,

Beury, D., Hot, D., Dubuquoy, L., Launay, D., Duflos, G., Body-Malapel, M., 2022. Oral exposure to polyethylene microplastics alters gut morphology, immune response, and microbiota composition in mice. Environ. Res. 212 (Pt B), 113230 https://doi.org/10.1016/j.envres.2022.113230.

Domenech, J., Hern´andez, A., Rubio, L., Marcos, R., Cort´es, C., 2020. Interactions of polystyrene nanoplastics with *in vitro* models of the human intestinal barrier. Arch. Toxicol. 94 (9), 2997–3012. https://doi.org/10.1007/s00204-020-02805-3.

Eliso, M.C., Bergami, E., Manfra, L., Spagnuolo, A., Corsi, I., 2020. Toxicity of nanoplastics during the embryogenesis of the ascidian *Ciona robusta* (Phylum Chordata). Nanotoxicology 14 (10), 1415–1431. https://doi.org/10.1080/17435390.2020.1838650.

Frijhoff, J., Winyard, P.G., Zarkovic, N., Davies, S.S., Stocker, R., Cheng, D., Knight, A.R., Taylor, E.L., Oettrich, J., Ruskovska, T., Gasparovic, A.C., Cuadrado, A., Weber, D., Poulsen, H.E., Grune, T., Schmidt, H.H., Ghezzi, P., 2015. Clinical relevance of biomarkers of oxidative stress. Antioxidants Redox Signal. 23 (14), 1144–1170. https://doi.org/10.1089/ars.2015.6317.

Halimu, G., Zhang, Q., Liu, L., Zhang, Z., Wang, X., Gu, W., Zhang, B., Dai, Y., Zhang, H., Zhang, C., Xu, M., 2022. Toxic effects of nanoplastics with different sizes and surface charges on epithelial-to-mesenchymal transition in A549 cells and the potential toxicological mechanism. J. Hazard Mater. 430, 128485 https://doi.org/10.1016/j. jhazmat.2022.128485.

Huang, J., Dong, G., Liang, M., Wu, X., Xian, M., An, Y., Zhan, J., Xu, L., Xu, J., Sun, W., Chen, S., Chen, C., Liu, T., 2022. Toxicity of micro(nano)plastics with different size and surface charge on human nasal epithelial cells and rats via intranasal exposure. Chemosphere 307 (Pt 4), 136093. https://doi.org/10.1016/j. chemosphere.2022.136093.

Ivleva, N.P., 2021. Chemical analysis of microplastics and nanoplastics: challenges, advanced methods, and perspectives. Chem. Rev. 121 (19), 11886–11936. https:// doi.org/10.1021/acs.chemrev.1c00178.

Karakolis, E.G., Nguyen, B., You, J.B., Rochman, C.M., Sinton, D., 2019. Fluorescent dyes for visualizing microplastic particles and fibers in laboratory-based studies. Environ. Sci. Technol. Lett. 6, 334–340. https://doi.org/10.1021/acs.estlett.9b00241.

Kik, K., Bukowska, B., Sicin´ska, P., 2020. Polystyrene nanoparticles: sources, occurrence in the environment, distribution in tissues, accumulation, and toxicity to various organisms. Environ. Pollut. 262. https://doi.org/10.1016/j.envpol.2020.114297.

Kim, S.A., Kim, L., Kim, T.H., An, Y.J., 2022. Assessing the size-dependent effects of microplastics on zebrafish larvae through fish lateral line system and gut damage. Mar. Pollut. Bull. 185 (Pt A), 114279 https://doi.org/10.1016/j. marpolbul.2022.114279.

Lesniak, A., Salvati, A., Santos-Martinez, M.J., Radomski, M.W., Dawson, K.A., Åberg, C., 2013. Nanoparticle adhesion to the cell membrane and its effect on nanoparticle uptake efficiency. J. Am. Chem. Soc. 135 (4), 1438–1444. https://doi.org/10.1021/ ja309812z.

Liu, S., Li, Y., Shang, L., Yin, J., Qian, Z., Chen, C., Yang, Y., 2022. Size-dependent neurotoxicity of micro- and nanoplastics in flowing condition based on an in vitro microfluidic study. Chemosphere 303 (Pt 3), 135280. https://doi.org/10.1016/j. chemosphere.2022.135280.

Mamun, A.A., Prasetya, T.A.E., Dewi, I.R., Ahmad, M., 2023. Microplastics in human food chains: food becoming a threat to health safety. Sci. Total Environ. 858 (Pt 1), 159834 https://doi.org/10.1016/j.scitotenv.2022.159834.

Nguyen, B., Tufenkji, N., 2022. Single-particle resolution fluorescence microscopy of nanoplastics. Environ. Sci. Technol. 56 (10), 6426–6435. https://doi.org/10.1021/ acs.est.1c08480.

Qiao, R., Mortimer, M., Richter, J., Rani-Borges, B., Yu, Z., Heinlaan, M., Lin, S.,

Ivask, A., 2022. Hazard of polystyrene micro-and nanospheres to selected aquatic and terrestrial organisms. Sci. Total Environ. 853, 158560 https://doi.org/10.1016/ j.scitotenv.2022.158560.

Ramsperger, A.F.R.M., Bergamaschi, E., Panizzolo, M., Fenoglio, I., Barbero, F., Peters, R., Undas, A., Purker, S., Giese, B., Lalyer, C.R., Tamargo, A., Moreno- Arribas, M.V., Grossart, H.P., Kühnel, D., Dietrich, J., Paulsen, F., Afanou, A.K., Zienolddiny-Narui, S., Eriksen Hammer, S., Kringlen Ervik, T., Graff, P., Brinchmann, B.C., Nordby, K.C., Wallin, H., Nassi, M., Benetti, F., Zanella, M.,

Brehm, J., Kress, H., Lo¨der, M.G.J., 2022. Laforsch CNano- and microplastics: a comprehensive review on their exposure routes, translocation, and fate in humans. NanoImpact 29, 100441. https://doi.org/10.1016/j.impact.2022.100441.

Rubio, L., Marcos, R., Herna´ndez, A., 2020a. Potential adverse health effects of ingested micro- and nanoplastics on humans. Lessons learned from in vivo and in vitro mammalian models. J. Toxicol. Environ. Health B Crit. Rev. 23 (2), 51–68. https:// doi.org/10.1080/10937404.2019.1700598.

Rubio, L., Barguilla, I., Domenech, J., Marcos, R., Herna´ndez, A., 2020b. Biological effects, including oxidative stress and genotoxic damage, of polystyrene nanoparticles in different human hematopoietic cell lines. J. Hazard Mater. 398, 22900 https://doi.org/10.1016/j.jhazmat.2020.122900.

Sawyer, L., Grubb, D.T., Meyers, G.F., 2008. Polymer Microscopy. Springer Science and Business Medium, New York. https://doi.org/10.1007/978-0-387-72628-1.

Shadel, G.S., Horvath, T.L., 2015. Mitochondrial ROS signaling in organismal homeostasis. Cell 163, 560–569. https://doi.org/10.1016/j.cell.2015.10.001.

Tenuta, T., Monopoli, M.P., Kim, J., Salvati, A., Dawson, K.A., Sandin, P., Lynch, I., 2011. Elution of labile fluorescent dye from nanoparticles during biological use. PLoS One 6 (10), e25556. https://doi.org/10.1371/journal.pone.0025556.

Tursi, A., Baratta, M., Easton, T., Chatzisymeon, E., Chidichimo, F., De Biase, M., De Filpo, G., 2022. Microplastics in aquatic systems, a comprehensive review: origination, accumulation, impact, and removal technologies. RSC Adv. 12 (44), 28318–28340. https://doi.org/10.1039/d2ra04713f.

Wang, Q., Bai, J., Ning, B., Fan, L., Sun, T., Fang, Y., Wu, J., Li, S., Duan, C., Zhang, Y., Liang, J., Gao, Z., 2020. Effects of bisphenol A and nanoscale and microscale polystyrene plastic exposure on particle uptake and toxicity in human Caco-2 cells. Chemosphere 254, 126788. https://doi.org/10.1016/j.chemosphere.2020.126788.

Wu, B., Wu, X., Liu, S., Wang, Z., Chen, L., 2019. Size-dependent effects of polystyrene microplastics on cytotoxicity and efflux pump inhibition in human Caco-2 cells.

Chemosphere 221, 333–341. https://doi.org/10.1016/j.chemosphere.2019.01.056.

Wu, Y., Yao, Y., Bai, H., Shimizu, K., Li, R., Zhang, C., 2023. Investigation of pulmonary toxicity evaluation on mice exposed to polystyrene nanoplastics: the potential protective role of the antioxidant N-acetylcysteine. Sci. Total Environ., 158851 https://doi.org/10.1016/j.scitotenv.2022.158851, 855.

Xu, M., Halimu, G., Zhang, Q., Song, Y., Fu, X., Li, Y., Li, Y., Zhang, H., 2019.

Internalization and toxicity: a preliminary study of effects of nanoplastic particles on human lung epithelial cell. Sci. Total Environ. 694, 133794 https://doi.org/ 10.1016/j.scitotenv.2019.133794.

Xu, C., Zhang, B., Gu, C., Shen, C., Yin, S., Aamir, M., Li, F., 2020. Are we underestimating the sources of microplastic pollution in terrestrial environment?

J. Hazard Mater. 400, 123228 https://doi.org/10.1016/j.jhazmat.2020.123228.

Yang, S., Cheng, Y., Chen, Z., Liu, T., Yin, L., Pu, Y., Liang, G., 2021. *In vitro* evaluation of nanoplastics using human lung epithelial cells, microarray analysis and co-culture model. Ecotoxicol. Environ. Saf. 226, 112837 https://doi.org/10.1016/j. ecoenv.2021.112837.

Yong, C.Q.Y., Valiyaveettil, S., Tang, B.L., 2020. Toxicity of microplastics and nanoplastics in mammalian systems. Int. J. Environ. Res. Publ. Health 17 (5), 1509. https://doi.org/10.3390/ijerph17051509.

Zhang, Y.X., Wang, M., Yang, L., Pan, K., Miao, A.J., 2022. Bioaccumulation of differently sized polystyrene nanoplastics by human lung and intestine cells.

J. Hazard Mater. 439, 129585 https://doi.org/10.1016/j.jhazmat.2022.129585.

Zhou, N., Wang, Z., Yang, L., Zhou, W., Qin, Z., Zhang, H., 2023. Size-dependent toxicological effects of polystyrene microplastics in the shrimp *Litopenaeus vannamei* using a histomorphology, microbiome, and metabolic approach. Environ. Pollut. 316 (Pt 2), 120635 https://doi.org/10.1016/j.envpol.2022.120635

ANNEXES

ANNEXES

7.1.1. Supplementary figures related to the published paper entitled Hazard assessment of different-sized polystyrene nanoplastics in hematopoietic human cell lines

Hazard assessment of different-sized polystyrene nanoplastics in three hematopoietic human cells

Alireza Tavakolpour[1,*], Balasubramanyam Annangi[1,*], Aliro Villacorta[1,2], Gooya Banaei[1], Juan Martin[1], Susana Pastor[1], Ricard Marcos[1,*], Alba Hernández[1,*]

[1]Group of Mutagenesis, Department of Genetics and Microbiology, Faculty of Biosciences, Universitat Autònoma de Barcelona, Cerdanyola del Vallès (Barcelona), Spain.

[2]Facultad de Recursos Naturales Renovables, Universidad Arturo Prat, Iquique, Chile.

**both authors contribute equally.*

SUPPLEMENTARY FIGURES

ANNEXES

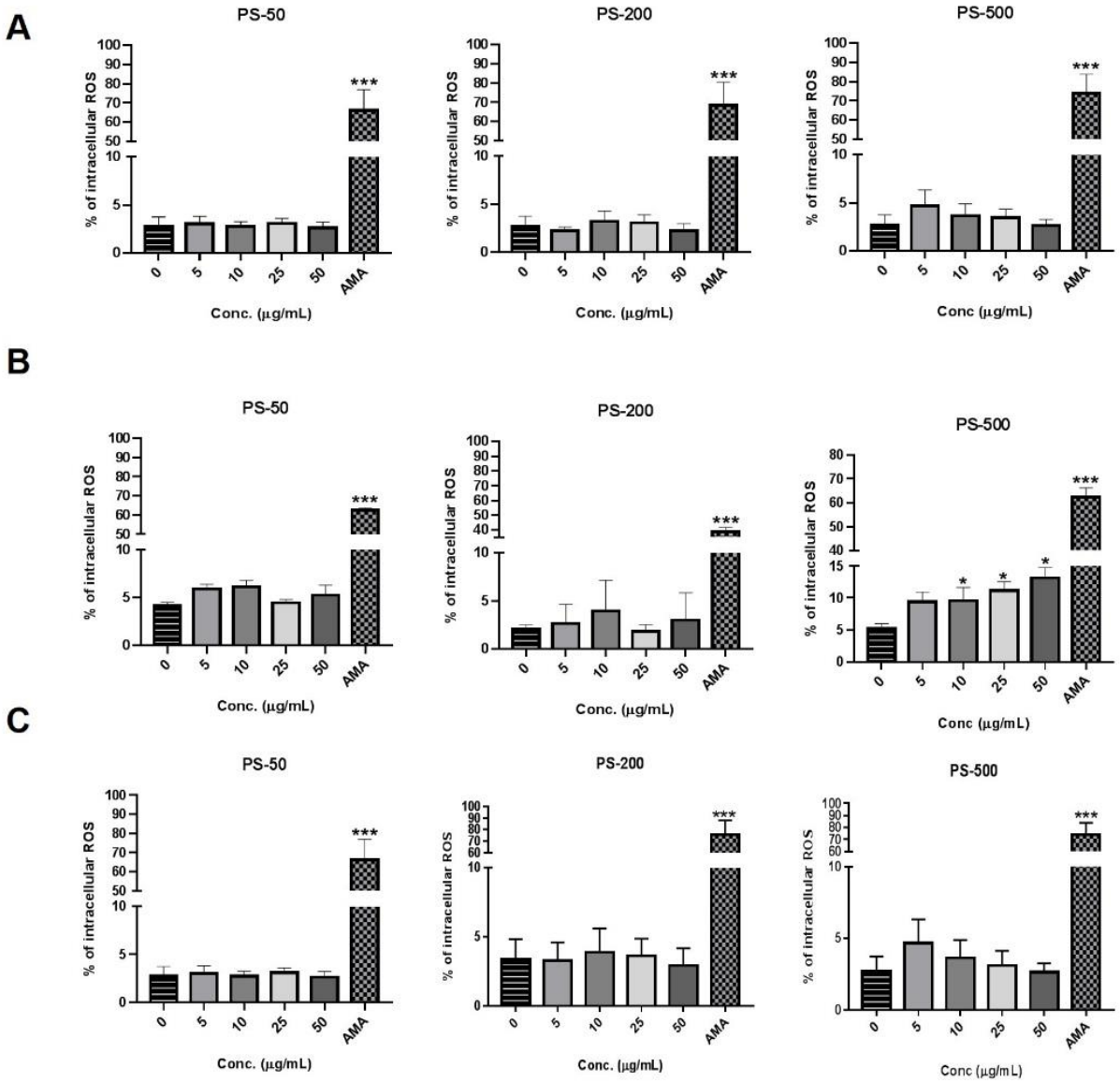


Figure S1. The histograms show the percentage of intracellular reactive oxygen species due to exposure of different sizes of PSNPLs viz., PS-50, PS-200, and PS-500 in Raji-B (A), THP-1 (B) and TK6 cells (C) after 24 h of treatments. Data are represented as mean ± SEM. *$P < 0.05$ ***$P < 0.001$ (2-way ANOVA and student's *t*-test).

ANNEXES

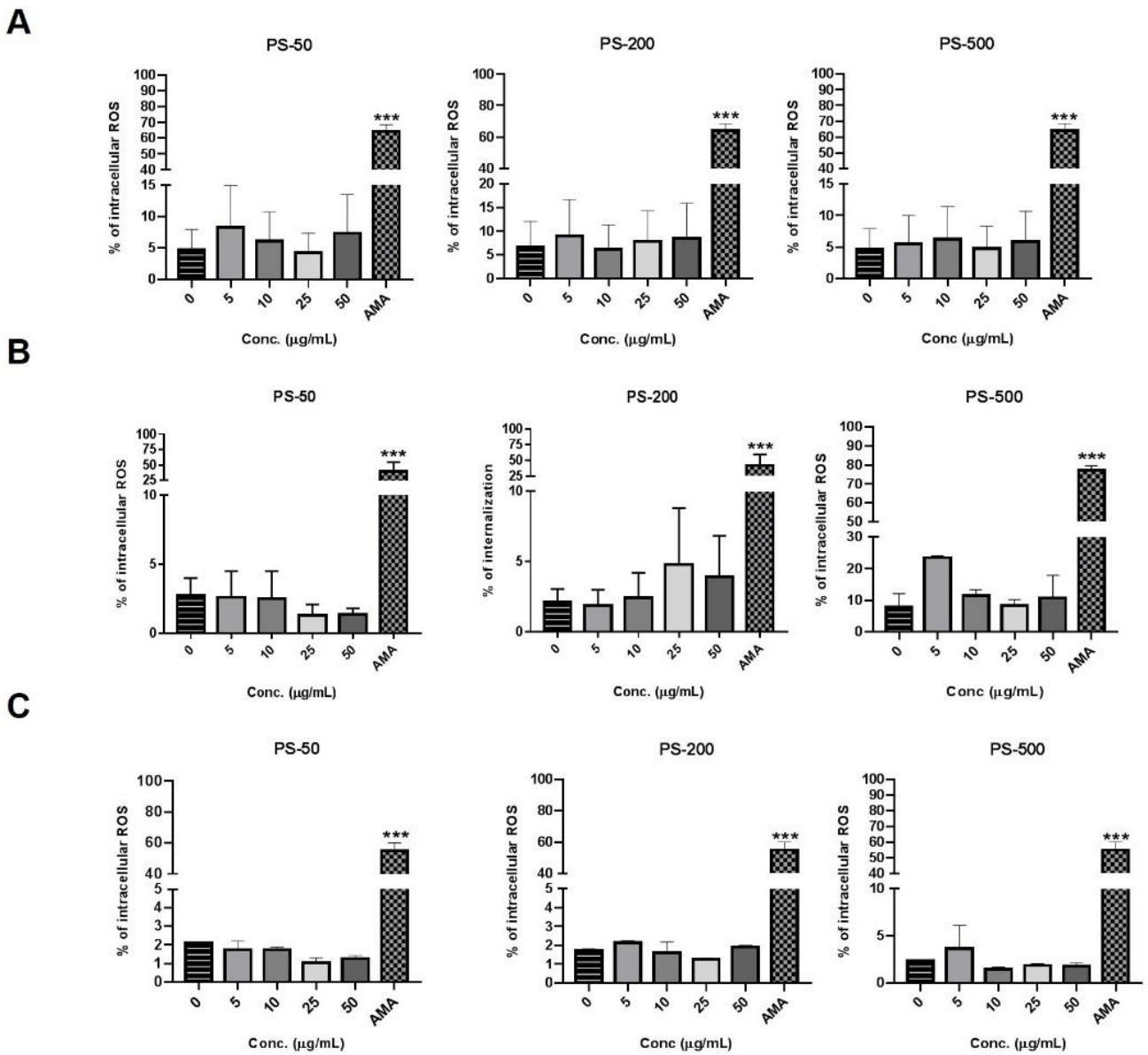


Figure S2. The histograms show the percentage of intracellular reactive oxygen species due to exposure of different sizes of PSNPLs viz., PS-50, PS-200, and PS-500 in Raji-B (A), THP-1 (B) and TK6 cells (C) after 48 h of treatments. Data are represented as mean ± SEM. ****P* < 0.001 (2-way ANOVA and student's t-test).

ANNEXES

ANNEXES

7.2. The pdf of the published paper entitled Harmful effects of true-to-life nanoplastics derived from PET water bottles in alveolar macrophages

ANNEXES

Environmental Pollution 348 (2024) 123823

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Harmful effects of true-to-life nanoplastics derived from PET water bottles in human alveolar macrophages.☆

Alireza Tavakolpournegari [a], Aliro Villacorta [a,b], Michelle Morataya-Reyes [a], Jéssica Arribas Arranz [a], Gooya Banaei [a], Susana Pastor [a], Antonia Velázquez [a], Ricard Marcos [a,*], Alba Hernández [a], Balasubramanyam Annangi [a]

[a] Group of Mutagenesis, Department of Genetics and Microbiology, Faculty of Biosciences, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Barcelona, Spain
[b] Facultad de Recursos Naturales Renovables, Universidad Arturo Prat, Iquique, Chile

ARTICLE INFO

Keywords:
PET
MH-S cells
ROS
Mitochondrial membrane potential
Macrophage polarization

ABSTRACT

The increasing presence of secondary micro/nanoplastics (MNPLs) in the environment requires knowing if they represent a real health concern. To such end, an important point is to test representative MNPLs such as the denominated true-to-life MNPLs, resulting from the degradation of plastic goods in lab conditions. In this study, we have used polyethylene terephthalate (PET) NPLs resulting from the degradation of PET water bottles. Since inhalation is an important exposure route to environmental MNPLS, we have used mouse alveolar macrophages (MH-S) as a target cell, and the study focused only on the cells that have internalized them. This type of approach is novel as it may capture the realistic adverse effects of PETNPLs only in the internalized cells, thereby miti- gating any biases while assessing the risk of these MNPLs. Furthermore, the study utilized a set of biomarkers including intracellular reactive oxygen species (ROS) levels, variations on the mitochondrial membrane potential values, and the macrophage polarization to M1 (pro-inflammatory response) and M2 (anti-proinflammatory response) as possible cellular effects due to PETNPLs in only the cells that internalized PETNPLs. After exposures lasting for 3 and 24 h to a range of concentrations (0, 25, 50, and 100 μg/mL) the results indicate that no toxicity was induced despite the 100% internalization observed at the highest concentration. Significant intracellular levels of ROS were observed, mainly at exposures lasting for 24 h, in an indirect concentration-effect relation- ship. Interestingly, a reduction in the mitochondrial membrane potential was observed, but only at exposures lasting for 24 h, but without a clear concentration-effect relationship. Finally, PETNPL exposure shows a sig- nificant polarization from M0 to M1 and M2 subtypes. Polarization to M1 (pro-inflammatory stage) was more marked and occurred at both exposure times. Polarization to M2 (anti-inflammatory stage) was only observed after exposures lasting for 24 h. Due to the relevance of the described biomarkers, our results underscore the needfor further research, to better understand the health implications associated with MNPL exposure.

1. Introduction

Plastic pollution is an important environmental concern due to the ever-increasing utilization of plastic products in each sphere of human life. The presence of large amounts of plastic materials in nature would result in both physical and chemical or biological degradation into smaller particles such as micro and nano-plastics (MNPLs). Subse- quently, they are released into the environment eventually reaching different types of ecosystems and making them a global environmental concern (Rubio et al., 2020; Gigault et al., 2021; Kumar et al., 2022). Due to that, there has been a good scope of MNPLs presence in marine food web because of their ingestion, subsequently giving rise to the trophic transfer of MNPLs to humans with potential human health im- plications (Carbery et al., 2018). In agreement, a few recent studies evaluated the potential trophic transfer of nanoplastics in marine or-ganisms *via* a three-step food chain as well as reaching humans through trophic transfer. It was shown that exposure of microalgae (*Dunaliella salina*) to amine-modified nanopolystyrene was transferred to small

☆ This paper has been recommended for acceptance by Maria Cristina Fossi.

* Corresponding author. Group of Mutagenesis, Department of Genetics and Microbiology, Faculty of Biosciences, Universitat Autònoma de Barcelona, Campus of Bellaterra, 08193, Cerdanyola del Vallès, Barcelona, Spain.
E-mail address: ricard.marcos@uab.cat (R. Marcos).

https://doi.org/10.1016/j.envpol.2024.123823
Received 21 December 2023; Received in revised form 8 February 2024; Accepted 18 March 2024
Available online 19 March 2024

crustaceans (*Artemia franciscana)*, and to fish (*Larimichthys polyactis)*, eventually reaching higher trophic level organisms such as humans by the food chain. This resulted in the inhibition of digestive enzyme ac- tivity (α-amylase) in humans (Kim et al., 2022). Similarly, a food web accumulation modeling to simulate a lake ecosystem revealed the accumulation of microplastics (MPLs) *via the* food web in marine or- ganisms and higher trophic levels like humans prone to consumption of MPs contaminated seafood (Bhutto et al., 2023). Additionally, human exposure to MNPLs to humans could come from different plastic sources utilized commercially, they could reach the human system *via* various routes of exposure such as oral, inhalation, and dermal (Lim et al., 2021).

Potentially, the inhalation route of exposure to atmospheric MNPLs in humans could trigger oxidative stress and inflammation in lung tissue due to their translocation to the lungs, where the immune responses involving inflammatory cells like macrophages, in particular, human alveolar macrophages of the pulmonary system would be in direct contact and could internalize them *via* phagocytosis, thereby playing a crucial role in the immune defense of the human body. However, the functional aspects of macrophages could be affected if the exposure to MNPLs lasts for a long period (Allard et al., 2018; Chen et al., 2022; Jenner et al., 2022). Thus, the importance of investigating the impli- cations of MNPLs in human health specifically focusing on immune response at cellular and tissue levels is inevitable (Le et al., 2023). The polymeric plastics that are found in the environment are made up of different chemical polymers like polyethylene (PE), polyvinyl chloride (PVC), polystyrene (PS), polypropylene (PP), and polyethylene tere- phthalate (PET) (Tursi et al., 2022). Among these, PET is the most widely used plastic material for manufacturing plastic bottles e.g., water storage and transport bottles (Villacorta et al., 2022). Hence, their fate in the atmosphere and their possibility to reach the human body,including the respiratory system, is a matter of growing environmental and human health concern (Annangi et al., 2023). Accordingly, several studies have utilized macrophages of different tissular origin to show the potential of MNPLs in eliciting inflammatory responses after their exposure under *in vitro* conditions (Collin-Faure et al., 2022; Wang et al., 2023). Thus, MNPLs released from plastic food packaging (mainly polypropylene and PET) were easily engulfed by alveolar macrophage RAW264.7 cells inducing inflammation (MPLs) and suppressing the lysosomal activity (NPLs) (Deng et al., 2022). In addition, lipid meta- bolism alterations in human M1 macrophages exposed to polypropylene and polyvinyl chloride NPLs were observed, affecting oxidative stress and phagocytosis mechanisms (Zingaro et al., 2023). In a most recent finding (Washihira et al., 2023), PETNPLs were able to trigger an im- mune response as there was a significant increase in inflammatory cytokine secretion after their treatment of human macrophages. Nevertheless, while the mentioned studies did focus on the immunotoxic effects of MNPLs in general, none of them had considered the cellular effects of PETNPLs in subpopulations of macrophages with and without MNPL internalization. Moreover, there is a lack of comprehensive studies showing the subtle toxic effects in sorted populations of mac- rophages with PETNPLs among the total exposed macrophage popula- tion. Hence, we investigated the specific hazard effects of these nanoplastics by sorting and monitoring only the macrophages contain- ing PETNPLs, we regarded them as a target population among the totalexposed cells.

In this study, mouse alveolar macrophages (MH-S cells) were used to assess the potential adverse effects associated with true-to-life PETNPL exposure in only the cells that internalized them, as compared to the subpopulation without cellular internalization. This study considered different concentrations of PETNPLs. Furthermore, we sorted the cells specifically based on the PETNPLs internalization using the cell sorter technique, later the sorted cells with PETNPLs were studied for their cellular effects such as the increase in reactive oxygen species (ROS),loss of mitochondrial membrane potential, as well as the macrophagic polarization into important subtypes. It must be remembered that to fulfill their functional roles, macrophages undergo polarization to the classically (M1) and alternatively activated (M2) subtypes; M1 macro- phages are capable of pro-inflammatory responses, and M2 macro- phages of anti-proinflammatory responses. Thus, determining if MNPLs can modulate such polarizations is of great relevance. The selected cells are regarded as model cells for toxicological and immunological studies, including those induced by MNPLs (Delaney et al., 2023). Several studies have used macrophages to show the *in vitro* potential of MNPLs to cause inflammatory responses, polarization of macrophages into different sub- types, DNA damage, as well as loss of mitochondrial membrane potential (Pulvirenti et al., 2022; Koner et al., 2023; Wanget al., 2023).

In summary, the hypothesis of the study was to understand whether cell sorting based on the internalized PETNPLs could aid in estimatingthe subtle or sub-toxic effects in the sorted cells otherwise these changes may go unnoticed as compared to the total unsorted exposed population of cells. For that, our main aim was to assess the cellular changes observed in those cells that internalized PETNPLs, in comparison with the total exposed population, at different concentrations and time intervals.

2. Materials and methods

2.1. Obtention, labeling, and characterization of PETNPLs

Laboratory-manufactured PETNPLs were prepared using the method described by Villacorta et al. (2022). In brief, commercially available PET plastic water bottles underwent sanding using a diamond rotaryburr. The resulting powder was sieved through a 0.20 mm mesh and subsequently, 4 g of this material were combined with 40 mL of 90% trifluoroacetic acid (TFA) preheated to 50 °C. The mixture was stirred continuously at 200 rpm on a heated stirring plate (Heidolph In- struments GmbH & Co. KG, Schwabach, Germany) for 2 h, followed by agitation at room temperature overnight, and 40 mL of TFA 20% was added to the suspension. After the removal of large agglomerates/ag- gregates, the remaining volume was divided into glass tubes and centrifuged at 2500 relative centrifugal force (RCF) for 1 h. The resulting pellet was resuspended in 400 mL of 0.50% sodium dodecyl sulfate (SDS), subjected to sonication, and then transferred to 250 mL beakers and subjected to sonication on 9/9 s intervals of sonication and breaks for 2 min, and then transferred to a graded cylinder, allowing the larger fraction to settle for 1 h. The top 100 mL from each cylinder were collected and washed twice with water and pure ethanol, and the resulting pellet was resuspended in Milli-Q water at the desired con- centrations. After sonication, the suspension was stored at −80 °C, and ready-to-use aliquots were prepared by using the Nanogenotox protocol (Nanogenotox project, 2011).

The labeling of PETNPLs with a commercial fluorescent dye was accomplished by following the adapted protocol previously publishedfor both commercial (Tavakolpournegari et al., 2023) and lab-made plastics (Annangi et al., 2023). Such protocol was based on the studies previously reported (Karakolis et al., 2019; Nguyen and Tufenkji, 2022). In summary, a working solution comprising 1 mL of PETNPLs at a concentration of 5 mg/mL was introduced into a 1.5 mL tube containing 0.01 g of iDye Poly Pink (hereafter referred to as iDye). The resultant mixture was vortexed and then incubated at 70 °C for 1 h, later the mixture was allowed to cool down to room temperature and transferred to a falcon tube containing 9 mL of Milli-Q water. The resulting 10 mL of solution was centrifuged at 4000 rpm and filtered to eliminate the excess iDye. The PETNPLs labeled with iDye were subsequently resuspended in 10 mL of Milli-Q water, subjected to centrifugation at 4000 rpm twice,and finally resuspended in 1 mL of Milli-Q water. Thus, labeled iDye-PETNPLs were stored in light-protected conditions at 4 °C.

To evaluate the characteristics of the generated PETNPLs, scanning electron microscopy was performed on a Zeiss Merlin scanning electron microscopy (SEM) system (SEM Zeiss Merlin, Zeiss, Oberkochen,

Germany), following the protocol previously reported (Villacorta et al., 2023). Briefly, a working solution of the particle suspension at a concentration of 100 μg/mL was prepared. A 20 μL drop was placed on a silicon holder of 5 × 5 mm (Ted Pella, INC. Altadena California) and let dry covered under the laminar hood. For better contrast, samples were sputter-coated with a gold/palladium thin layer in an Emitech K550X from Quorum (Laughton, East Sussex, United Kingdom) as described (Domenech et al., 2020). Particles were analyzed by taking micrographs from random fields and counting a minimum of 1000 particles. To evaluate the hydrodynamic behavior of a solid colloidal spherical structure corresponding to our sample, the hydrodynamic behavior of the particle suspension was measured on water and on RPMI-supplemented media previously described on a Zetasizer® Ultra
Red from Malvern Panalytical (Cambridge, United Kingdom). A collection angle of 174.7° was used for DLS. The refraction index of PET used for this study was 1.57 while for water was 1.33 and 1.34 for supple- mented RPMI. The Z-potential was also evaluated using a DTS 1070 cuvette. For determining the lambda emission of our particle suspen- sions, particles were allowed to aggregate on a glass slide before observation. Slides were washed with methanol under a BSL2 laminar flow cabin and once the methanol evaporated a 20 μ L drop ofiDyePET-NPLs was deposited on the surface and a coverslip wasimmediately placed. A minimum of 15 min prior to observation was waited before the samples were studied on a Zeiss LSM 980 confocal system (Zeiss, Oberkochen, Germany). The selected excitation wave- length was 561 nm, and the emission was collected from 580 to 770 nm. For all cases, the data was arranged and analyzed using GraphPad prism
8.0 (GraphPad, San Diego, CA) and images were prepared using ImageJ software 1.8.0_172.

2.2. Cell culture

The mouse alveolar macrophage cell line (MH-S) utilized in this study was a generous gift by Dr. José Domínguez from the Innovation in Respiratory Infections and Tuberculosis Diagnosis Group at the Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Barcelona, Spain. The MH-S cells were cultured in RPMI-1640 medium (supplied by Biowest, lnc.), which was enriched with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2.5 μg/mL of Plasmocin. The cells were incubated at a temperature of 37 °C in an environment containing 5% CO_2. The cell culture medium was changed with fresh pre-warmed medium every 2–3 days.

2.3. MH-S cells treatment with PETNPLs

Cells were treated with various concentrations of PETNPLs sus- pended in RPMI-1640 medium to investigate their potential toxic ef- fects. Different cell densities were seeded for each type of assay, as further detailed in the corresponding section. After seeding, the cells were exposed to different concentrations of PETNPLs (0, 25, 50, and100 μg/mL) for 3 and 24 h, based on the endpoints of the study. We regarded these concentrations as the first starting point to know any significant decreases in cell viability. This would reveal the sub-toxic concentrations at which the cells would withstand the PETNPLs- induced cell death, thereby subtle changes or sub-toxic cellular func- tions could be investigated. A similar range of different concentrationsof MNPLs was selected by Yang et al. (2021) to assess the human lung response after their exposure. Furthermore, the shorter duration of ex- posures was based on the cellular internalization of PETNPLs in mac- rophages, at these treatment time points, PETNPLs may potentiate acute cellular effects, particularly in the sorted cells with PETNPLs. Taken together, the evaluation of the adverse effects of PETNPLs in the sorted cells was acute.

2.4. Cell sorting and isolation of iDye-PETNPLs internalized cells

For the specific objective of visualizing iDye-PETNPLs internaliza- tion, when using confocal microscopy, exposed cells were sorted usingthe cell sorter (FACSJazz cell sorter, Becton Dickinson, USA). Both, cells with internalized PETNPLs (positive) and without (negative) were sor- ted and suspended in RPMI-1640 medium based on the fluorescence intensity of iDye-PETNPLs at 585 nm emission (10,000 events recorded and gated using BD FACJazz software). To confirm the internalization of PETNPLs in sorted cells, confocal microscopy was used. For this purpose, a total of 80,000 sorted cells were seeded in microwell dishes with glass bottoms (MatTek, Ashland, OR, USA) and immediately assessed by confocal microscopy. The nuclei and cell membrane were stained with Hoechst dye (ThermoFisher Scientific, Carlsbad, CA, USA) at a dilutionof 1:500 and Cellmask™ Deep Red plasma (ThermoFisher Scientific, Carlsbad, CA, USA) at a dilution of 1:500, respectively. This staining process takes place for 15 min at room temperature. To visualize the iDye-PETNPLs within cells the emission wavelength of 585 nm was used. It was achieved using a Leica TCS SP5 confocal microscope. Multiple fields were randomly chosen from each sample. The acquired imageswere then processed utilizing ImageJ software with the Fiji extension.

2.5. Cell viability

MH-S cells were collected by centrifugation (100 rcf for 7 min) and resuspended in RPMI-1640 medium to reach the density of 2.5 10^5 cells/mL and then seeded in a 12-well plate. Exposure to PETNPLs lasts for 24 h with a range of concentrations (0, 25, 50, and 100 μg/mL. After exposure, the cells were diluted with Isoton solution (1:200) and
counted with ZTM series Coulter counter (Beckman Counter lnc., CA). The derived percentage values were calculated as the mean count ofcells for each treatment, relative to the values from the untreated control cells.

2.6. Intracellular ROS induction assessment

The ability of PETNPLs to produce reactive oxygen species (ROS) in MH-S cells was assessed by using the DCFH-DA (2′,7′-dichlorodihydro-fluorescein diacetate) method using flow cytometry. To be sure that ROS was measured only in the cells internalizing PETNPL, the MH-S cells were exposed to the iDye-PETNPLs (0, 25, 50, and 100 μg/mL) for 3 and 24 h. After exposure, the cells were detached by using trypsin (0.05%), centrifuged at 200 rcf for 10 min, pelleted, and then suspended in 10 μM of DCFH-DA at 37 °C for 30 min. The exposed cells were washed with PBS and analyzed by flow cytometry (CytoFlex, Beckman Coulter, USA). Hydrogen peroxide (H_2O_2, 1 mM) was used as a positive control. Cells containing PETNPLs were gated based on the fluorescent intensity ofiDye-PETNPLs at 585 nm emission, and the fluorescent intensity of DCFH-DA was assessed in the gated population at 530 nm emission. 10,000 cells per sample were scored and analyzed with the Cytexpert software.

2.7. Measurement of mitochondrial membrane potential

The loss of mitochondrial membrane potential (MMP) in MH-S cells due to PETNL exposure was measured by using the TMRM Mitoprobassay in the gated cells using flow cytometry. Cells were exposed to iDye-PETNPLs (0, 25, 50, and 100 μg/mL) for 3 and 24 h. After exposure, cells were pelleted and treated with 20 nM of TMRM prob for 30 min at37 °C. The exposed cells were washed with PBS and analyzed by flow cytometry (CytoFlex, Beckman Coulter, USA). Carbonyl cyanide m-
chlorophenyl hydrazone (CCCP, 5 μM) was used as a positive control and incubated for 5 min at 37 °C. By using flow cytometry, those cells containing PETNPLs were gated based on the fluorescent intensity of the iDye-staining with the excitation-emission of 561–630 nm respectively, and the fluorescent intensity of TMRM prob was also assessed in the

gated population with the excitation-emission of 488‑560 nm respectively. About 10,000 cells per sample were scored and analyzed with Cytexpert software.

2.8. Macrophage polarization assay by flow cytometry (FC)

One of the important responses of the macrophages, as immune cells, to the environmental agents is their polarization from M0 to M1 (pro-inflammatory subtype) and M2 (anti-inflammatory subtype) which would indicate their sensitivity to external stimuli (Fuchs et al., 2016). Accordingly, determining if PETNPLs trigger macrophagic polarizationis something to be known. For that, macrophage polarization assessment was carried out by using FITC fluorescent conjugated anti-CD86 and anti-CD163 monoclonal antibodies (Thermofisher Scientific, USA) to detect M1 and M2 polarized macrophages, respectively. Cells were exposed to iDye-PETNPLs (0, 25, 50, and 100 μg/mL) for 3 and 24 h. After exposure, cells were pelleted and treated with concentrations of500 μg/mL and 250 μg/mL of CD86 and CD163, respectively, for 30 min
at 4 °C. The exposed cells were washed with PBS and analyzed by flow cytometry (CytoFlex, Beckman Coulter, USA). LPS at the concentrationof 10 μg/mL was used as a positive control by treating the cells for 24 h. By cytometry, cells containing PETNPLs were gated based on the fluo- rescent intensity of the iDye-stain with the excitation-emission of561‑630. The fluorescent intensity of antibodies was evaluated in the gated population with the excitation-emission of 488‑520 nm for CD86 and the excitation-emission of 405‑436 nm for CD163 (in separate ex- periments for each antibody). The amount of 10,000 cells per samplewas scored and analyzed by using the Cytexpert software.

2.9. Macrophage polarization assay by confocal microscopy

To complement flow cytometry data, macrophage polarization was assessed by using confocal microscopy. To proceed, cells were seeded ina 6-well glass bottom dish with a density of 80,000 cells/well and treated with iDye-PETNPLs for 24 h (100 μg/mL). After exposure, cells were washed thrice with cold PBS and fixed with 4% paraformaldehyde incubated at 4 °C for 10 min. Later, cells were incubated with a blocking buffer (1% bovine serum albumin) for 45 min at 4 °C. Following, anti- CD86 and anti-CD163 antibodies were added and incubated for 2 h at room temperature. Finally, cells were washed twice with PBS, and
phalloidin (0.5%) was added to the cells to stain the cytoskeleton. The excess stain was removed by rinsing with PBS. Nuclei were stained with Hoechst dye (ThermoFisher Scientific, Carlsbad, CA, USA) at a dilutionof 1:500 for 15 min at room temperature. To visualize iDye-PETNPLs, CD86, CD163, and phalloidin within the cells, the emission wave- lengths of 585, 520, 436, and 488 nm were used, respectively. This visualization was achieved using a Zeiss LSM 980 confocal microscope. Multiple fields were randomly chosen from each sample. The acquired images were then processed utilizing ImageJ software with the Fiji extension.

2.10. Statistical analysis

Data, represented as the mean ± SEM values, were derived from three independent experiments, each of which was conducted byduplicate. For the statistical evaluation, a two-way ANOVA with Tur-key's multiple comparison tests was used, except where specifically noted otherwise. Significance levels were established as follows: *p 0.05, **p 0.01, and ***p ≤ 0.001. Data analysis was conducted usingGraphPad prism 8.0 (GraphPad, San Diego, CA).

3. Results and discussion

3.1. Characterization of PETNPLs and visualization of labeled PETNPLs

The size distribution and shape of PET nanoparticles in suspension were assessed using scanning electron microscopy for the dry state, anda Zetasizer device for the hydrodynamic behavior. The micrograph in Fig. 1a shows differences in particle morphology as well as in the polydispersity nature of the sample on water dispersion. The calculated average size of the particles in the dry state, as informed in Fig. 1b‑is slightly smaller (163.01 nm) than the size informed by DLS for water dispersion (292.00 nm), while the PDI value for both cases is never smaller than 3.9. This average size value (Z-average) is greater when RPMI is used for particle dispersion (1140 nm), which is a clear indicator of the presence of aggregates. The comparison of water dispersion and RPMI dispersion can be observed in Fig. 1c. One of the principal reasons for this increase in the hydrodynamic behavior may be the irregular shape of the particles, which may increase the contact surface interactions between particles and media components. The 0.3% of big aggregates detected by SEM (over 1 μm) could be highly represented due to the scattering effects or the increase of the suspension fraction behaving in this manner. The reported data are well represented by the correlation curves of the different measurements carried out, as depicted in Fig. 1d. These values, which could be interpreted as a huge increase in
the Z-average values, shifting from 292 nm to more than 1 μm agree with the shift observed for the Z-potential where a significant decrease on the absolute value from 26 to 8 is observed, indicating that the suspen- sion is more prone to form aggregates when the dispersion is made using supplemented culture media.
Regarding the labeled PETNPLs, the visualization was done by confocal microscopy, as observed in Fig. 1e, where the labeled PETNPLs are observed as green spots resulting from the presence of big particle clusters formed on the glass slide. Fig. 1f shows the emission spectra of these stained particles which peak from 610 to 620 nm. That peak emission agrees with the expected emission spectra of iDye which moves from 585 to 700 nm. In addition, no background fluorescence was observed indicating a lack of leaching, which all together indicates the suitability of the stained particles as a suitable model.

3.2. PETNPLs uptake detection by confocal microscopy, using sorted cells

Cell sorting by flow cytometry has been classically used to select and isolate a desired population of cells, from an heterogenous culture. In our case, we used iDye-labeled PETNPL to detect light signals in specific wavelengths emitted by those target cells internalizing labeled PETNPLs. This cell-sorting approach has been used to isolate differentcell types internalizing or not fluorescent nanoparticles (Latroche et al., 2018; Ciaglia et al., 2019). The identification and isolation of cells containing PETNPLs were carried out by assessing the fluorescent intensity of the particles. Sub- sequently, those cells containing internalized PETNPLs were sorted and collected from the live cell population, previously exposed to three different concentrations (25, 50, and 100 μg/mL) of iDye-labeled PETNPL. The percentage of cells that internalized PETNPLs, from theoverall exposed cell population, is indicated in Supplementary Fig. S1. The internalization levels show a direct relationship with exposure concentrations where 100 percent of the cells exposed to the concen- tration of 100 μg/mL internalized PETNPLs. To confirm that all the positive gated cells carried internalized PETNPLs, positive cell sorting was carried out and confocal microscopy was applied to the selected cells. As observed in Fig. 2, the obtained confocal images confirmed that 100% of the sorted cells possessed intracellular iDye-PETNPLs. Inter- estingly, more signals were observed in those cells exposed to the highest concentration (100 μg/mL).

3.3. Cell viability in MH-S cells exposed to PETNPLs scored in sorted cells

To assess the potential cytotoxic effects of PETNPLs internalization, MH-S cells containing iDye-PETNPLs, were used. To proceed, positive MH-S cells were selected by sorting cells exposed to several concentra-tions (25, 50, and 100 μg/mL) for 24 h. As illustrated in Supplementary

ANNEXES

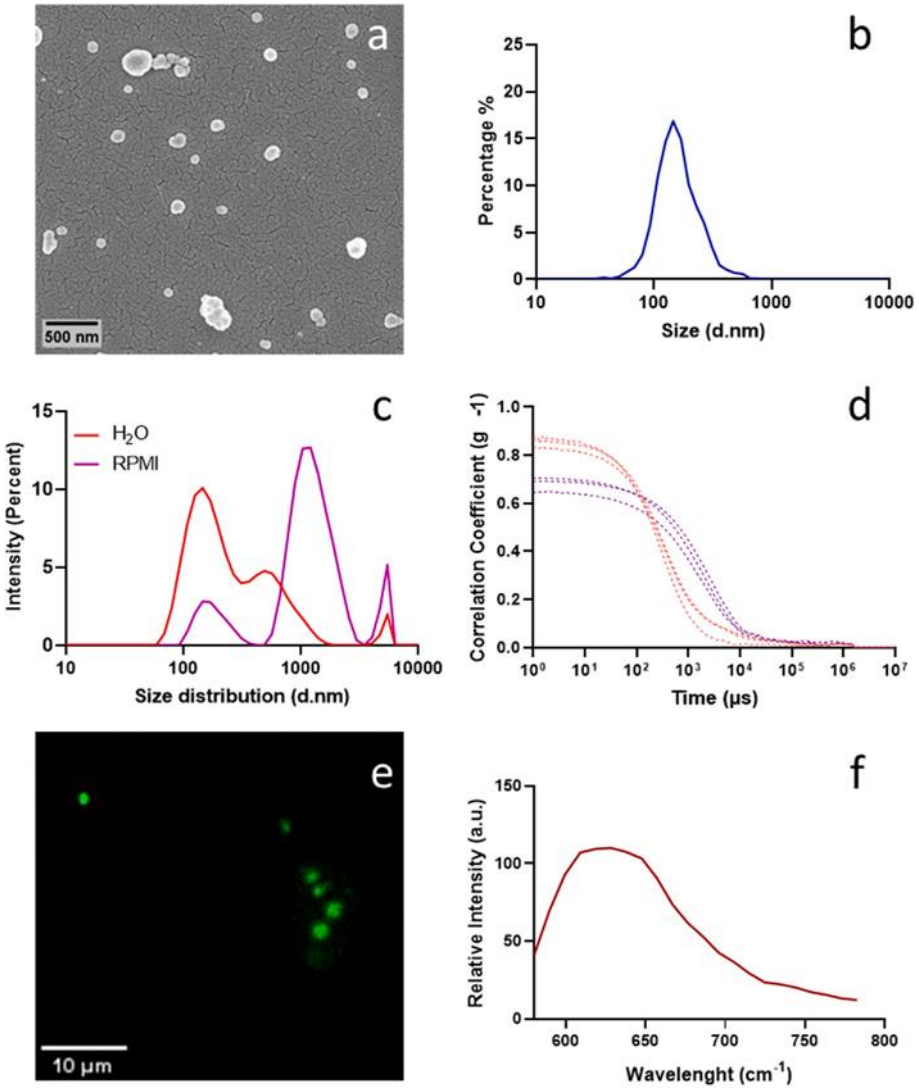


Fig. 1. (a) Characterization of PETNPLs morphology assessed by SEM. (b) Measured SEM size distribution of PETNPLs. (c) The intensity values for the hydrodynamic behavior of PETNPLS for both water (red) and media (purple) dispersion. (d) Correlation coefficients for (c) distribution data. (e, f) Visualization and fluorescent spectra confirmation for particle aggregates, as done by confocal microscopy and lambda scan, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. S2 no significant changes in the viability of MH-S cells that uptake PETNPLs were observed. Therefore, no toxic effects are associated with the internalization of PETNPLs. This response is like that observed in overall exposed cell populations (unsorted ones). Thus, human leukemia monocytic THP-1 cells exposed to PETNPLs reported no significant decrease in cell viability after 24 h of exposure (Villacorta et al., 2022). However, that study chose the total exposed THP-1 cells to assess the cytotoxic response unlike we evaluated only the sub-populations of cells with PETNLs, as compared to total exposed cells, which may give a more precise and robust risk assessment of the effects. However, our data revealed that there was also no significant decrease in cell viability in only a subpopulation of cells with PETNPLs, as compared to the total population. This may add value to the existing information about therisk assessment of MNPLs. Moreover, the obtained data, along with other studies, may not only suggest the significance of cell viability that would aid in identifying the sub-lethal concentrations of the MNPLs but also its relevance to evaluating the subtle toxic effects like changes in ROS, MMP, and polarization of macrophages in only the cells containing PETNPLs; otherwise, these sub-toxic effects may be masked in the total exposed population. A recent study by Annangi et al. (2023) demon-strated the sub-lethal effects, like an increase in ROS and loss of MMP, due to PETNPLs in primary human nasal epithelial (HNEpCs) cells although they did not observe any significant decreases in cell viabilityat the tested concentrations (0–100 μg/mL). In accordance, no signifi- cant changes in cell viability were observed in three different human cell types [(human intestinal epithelial (Caco-2), human hepatoma (HepG2), and human hepatic (HepaRG) cells] after exposure to PE, PP, PET, andPVC MPLs (Stock et al., 2021) despite the successful internalization observed. This may escape the subtle or sub-lethal cellular effects in the exposed cells having the internalized MNPLs, thereby underestimatingthe risk posed by them. Although this study utilized relatively high acute concentrations to assess the impacts of PETNPLs in the sorted cells, as compared to plausible environmentally relevant doses, a recent study estimated a significantly high mass of MPLs that could reach humans due to inges- tion based on a meta-analysis of 59 research papers. It reported that humans may ingest 0.2–10 mg/day/kg for 70 kg man, which would translate to 0.1 to 5 per week (Senathirajah et al., 2021). Nevertheless,the extrapolation of the doses used here to possible real-life exposure conditions is out of the scope of this study.

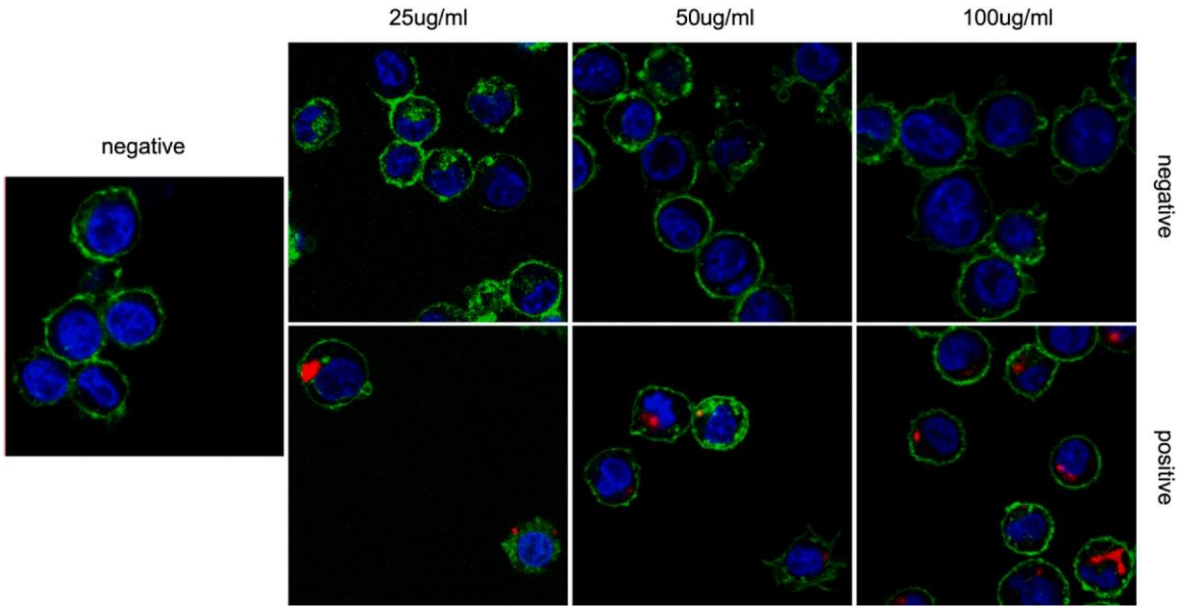


Fig. 2. Confocal microscopy images confirmed the presence of internalized PETNPLs in positive sorted MH-S cells. The red color represents the PETNPLs, and the green and blue colors represent the cell membrane and cell nuclei, respectively. No PETNPLs were observed neither in the negative sorted cells nor in the unexposed cells (0 μg/mL). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. *Induction of ROS by PETNPLs in gated cells*

To achieve an insight into the harmful effects of exposure toPETNPLs, from a biological perspective, various biomarkers can be used. Among them, ROS is one of the crucial indicators to identify cellular stress and toxicity in response to external components. This biomarker is widely recognized in different studies where an imbalance in the anti- oxidant levels results in increased ROS production, leading to DNA damage and even to cellular death (Jomova et al., 2023). In this study,the intracellular oxidant levels in the gated cells having internalized PETNPLs at 3 and 24 h post-treatment were measured using the DCFH-DA assay. Specifically, our data indicated that PETNPLs would possibly elevate the oxidant levels, as compared with untreated cells, in a time-dependent manner (Fig. 3) through a redox signaling mechanism, since the DCFH probe utilized in this study might not measure H_2O_2 and other ROS levels, rather its oxidation could be catalyzed by iron ions(Fe^{+2}) in the presence of oxygen or H_2O_2 to DCF, the fluorescent sub- product. Furthermore, utilizing the probe might not result in a quanti- tative measurement of the generation of ROS (Kalyanaraman et al., 2012). Moreover, we found that there were significant increases in the oxidant levels only at the highest concentration tested (100 mg/mL) at 3 h post-treatment, but the oxidant levels after 24 h were significant at all the tested concentrations (Fig. 3). Previous studies suggested PETNPLs could generate ROS albeit with a varying degree in different cell types, a slight increase in ROS levels in mouse alveolar macrophages (RAW 246.7) (Aguilar-Guzmán et al., 2022), and similar weak effects were reported in human alveolar macrophages where increased ROS levels were only observed in one of the concentrations tested (Zhang et al., 2022), but there were obvious increases in production of ROS in human nasal epithelial cells exposed to PETNPLs (Annangi et al., 2023). It must be pointed out that all these studies have also utilized DCFH-DA as a probe which could estimate the oxidant levels over the generation of ROS (Kalyanaraman et al., 2012), hence the generation of ROS byPETNPLs may be not representative in the studies while employing DCFH probe for the estimation of ROS. Moreover, our study also addressed the biases associated with these studies considering they estimated the oxidant levels in the whole heterogenous population of cells exposed to PETNPLs, while the present study analyzed the oxidant levels in those gated cells having internalized PETNPLs, providing more robust data.

3.5. *Loss of mitochondrial membrane potential in gated cells*

The MMP is one of the indicators of cell toxicity (Sakamuru et al., 2016). Since mitochondria have been considered as a target of MNPL action (Cortés et al., 2020; Lin et al., 2022; Tavakolpournegari et al.,

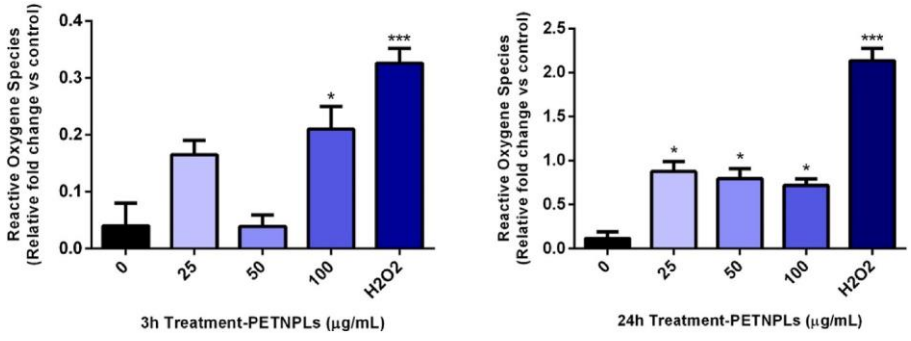


Fig. 3. The histograms show the fold change of ROS production in MH-S cells exposed to PETNPLs for 3 and 24 h at the concentrations of 0, 25, 50, and 100 μg/mL. Data are represented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$ (2-way ANOVA and Student's t-test).

2023), changes in MMP should be considered as a biomarker in those studies determining the potentially hazardous effects of MNPL exposure.As observed in Fig. 4, our results revealed significant decreases in MMP values in those MH-S cells containing PETNPLs after exposureslasting for 24 h. These findings are in line with previous studiesdemonstrating the ability of PETNPLs to disrupt MMP in different celllines, such as in adenocarcinoma human alveolar basal epithelial A549cells (Zhang et al., 2022), and in human nasal epithelial primary(HNEpCs) cells (Annangi et al., 2023). Collectively, these studies underlined the consistent effect of PETNPLs on MMP in various celltypes and reinforced the significance of our data in elucidating thespecific consequences of PETNPL exposure on the MH-S cells that haveinternalized them. The mitochondrial membrane potential, in associa-tion with other toxic responses of cells, could show the harmful effect ofenvironmental agents such as PETNPLs and, as discussed above,different cell types in the whole population of samples treated with MNPLs showed a significant change when assessed for the MMP. In this assay, we postulated that cells that internalized PETNPLs should show a remarkable change in MMP, concluding that PETNPLs can causeharmful effects on the mitochondria via MMP. It is important to pointout the relevance of the exposure time on the expression of this biomarker. Accordingly, exposures lasting for 3 h were too short to induce the expected effects, which were observed when exposures were extended to 24 h.

3.6. *Macrophage polarization by PETNPLs in gated cells*

The macrophage response to environmental insults can appear as their polarization towards two distinct phenotypes called M1 and M2that could act as pro-inflammatory and anti-inflammatory subtypes, respectively. This type of cellular response in macrophages could be a clue to identifying the immunotoxic effects of NPLs exposure in immune-based cells like MH-S cells (Pérez and Rius-Pérez, 2022; Wu et al., 2023). In our study, the potential of PETNPLs in modulating the immune responses of mouse alveolar macrophages (MH-S cells) was evaluated bystudying the toll-like receptors on the surface of the macrophages rep-resenting the polarized macrophage from M0 to M1 and M2 subtypes. The data indicated that the presence of PETNPLs in MH-S cells couldsignificantly affect the polarization of the MH-S cells from M0 to M1 and M2 subtypes at exposures (0, 25, 50, and 100 μg/mL) lasting for 3 and 24 h. Interestingly, we observed a very significant polarization of mac- rophages (M0) towards the M1 subtype in the gated cells containing PETNPLs, as evidenced by the increased expression of the structural surface biomarker CD86. Its expression decreased when the concentra- tion increased, in comparison with untreated cells at both exposure time points. This indicates the role of PETNPLs in eliciting a pro- inflammatory response (Fig. 5). Our results agree with the polariza- tion towards a pro-inflammatory phenotype observed in MH-S cells exposed to PSNPLs when assessed by using the CD38 biomarker (Col- lin-Faure et al., 2022). Moreover, as indicated in Fig. 5, there was a consistent level of M0 to M1 polarization between the two time points lasting for 3 - 24 h, since this effect was significantly observed in the sorted cells with internalized PETNPLs at both time points. Hence, at different time points, the effects of PETNPLs to polarize the macro- phages prominently to the M1 sub-type remained stable in the sorted cells with PETNPLs population. Unfortunately, no similar studies have been detected in the open literature to confirm the relevance of the exposure time, although the importance of time in macrophage polari- zation has been pointed out (Murray, 2017). Although without checking the presence of M1/M2 subtypes, indirect studies reporting the secretion of inflammatory cytokines in murine (RAW264.7) macrophages (Wanget al., 2023) and human lung macrophage cells (Washihira et al., 2023) after exposure to PS-MNPLs exposure suggest a role on macrophage polarization. Furthermore, a study using different NPLs with different sizes displayed strongly modulated cytokine secretion patterns, sug- gesting downregulation of the inflammatory phenotype indicative of M2 macrophage induction, and more towards a pro-inflammatory pheno-type i.e. M1 sub-type (Wolff et al., 2023).

Besides, our data also revealed that there was a polarizing effect towards the M2 subtype in the gated cells containing PETNPLs, as observed by the increased expression of CD163 surface biomarker when compared to the untreated cells. These effects were more evident after24 h of treatment at all concentrations tested suggesting the existence ofa subpopulation of macrophages (M2) in the total population involved in anti-inflammatory or wound-healing responses with the M1 subtype. The response behind the different M2 responses between 3 and 24 h of exposure might agree with the macrophage immune response toward the time; meaning that at the very beginning of recognizing external insults, the immune pro-inflammatory response of macrophages occurs, while later, and to reduce the effects of pro-inflammatory cytokines, the M2 phenotype of the macrophages would raise and neutralize the pro- inflammation outcomes (Murray, 2017). Similarly, an M2 polarization of rainbow trout macrophages (RT-HKM) exposed to PSNPLs was re-ported, inferring an anti-inflammatory response in these cells (Brandtset al., 2023), as well as observed in macrophages derived from human monocytes (Wolff et al., 2023). It is important to point out that using true-to-life PETMPLs in an *in vivo* mouse model, a recent study including RNA-seq analysis has demonstrated that exposure affects immune ho-meostasis (Harusato et al., 2023).

To complement the previous FC studies, the potential macrophage polarization resulting from PETNPL exposure was assessed by immu-nocytochemistry using confocal microscopy. As a complementary study, this approach was only performed by detecting CD86 in the cell mem- brane. The obtained data showed that the cells containing PETNPLs triggered a macrophagic polarization from M0 to M1 (CD86) subtype because of PETNPLs internalization at 100 μg/mL concentration after

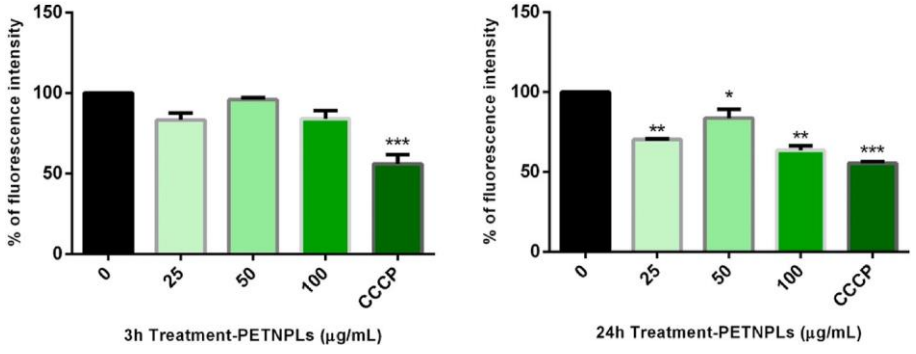


Fig. 4. The histograms show the effects of PETNPLs on the mitochondrial membrane potential of gated MH-S cells exposed for 3 and 24 h. The exposure lasting for24 h resulted in a significant decrease in the mitochondrial membrane potential, in comparison with the lack of effect of exposures lasting for 3 h. Data are rep- resented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (2-way ANOVA and Student's t-test).

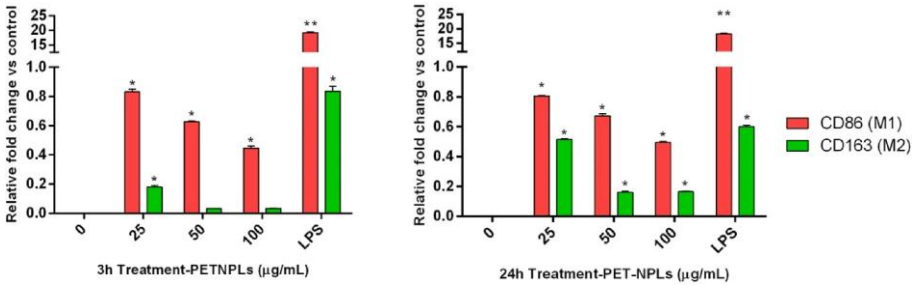


Fig. 5. The histograms indicate the influence of PETNPLs on the macrophage polarization from M0 to M1 and M2 subtypes in exposed MH-S cells. Data are represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, (2-way ANOVA and Student's t-test).

24 h of exposure (Fig. 6, a2).

From a global comprehensive point of view, the results describe the interconnection between MMP, ROS, and the immune response to PETNPL exposures. Alterations in MMP and ROS, not only are related to each other because the mitochondrial membrane potential acts as the main source of the intracellular ROS (Suski et al., 2018), but also considering the pathological issues caused by alterations in ROS and MMP balance that produce an inflammatory response. This idea is clear in the current study in a way that by looking at the obtained data, the harmonic trend between MMP, ROS, and M1/M2 macrophage polari- zation, even between the times of exposure and the concentrations, shows the connective outcome, as explained in the study of Wang et al. (2021) were changes in the mitochondrial membrane potential and the oxidative stress affected the regulation and activation of macrophage and immune system via different pathways.

The mitochondrial metabolism and the physiology of macrophages can be affected by different environmental factors and stimuli, whichallow to polarize of the macrophages into pro-inflammatory M1 phenotype or anti-inflammatory M2 phenotype (Xu et al., 2022). Mito- chondrial dysfunction such as loss of MMP may reprogram the energy metabolism as a decrease in mitochondrial oxidative metabolism, elevated uptake of glucose, and its breakdown *via* glycolysis (Bao et al., 2021). All this may lead to nitric oxide (NO) mediated accumulation of ROS which might prevent repolarization of M1 to M2 macrophages, skewing towards an inflammatory response (Bedard and Krause, 2007). However, by restoring the mitochondrial function the response from inflammatory to anti-inflammatory macrophages could be reversed (Van den Bossche et al., 2016).

4. Conclusion

The increasing levels of environmental plastic waste pollution require urgent studies to determine their potential toxicological profile, particularly regarding the byproducts resulting from their degradation (MNPLs). Since inhalation is an important exposure route to MNPLs, we have used mouse alveolar macrophages (MH-S) as an appropriate target. Aiming to evaluate representative MNPLs, we have used true-to-life PETNPLs obtained by sanding PET water bottles as an approach to those secondary MNPLs present in the environment. Finally, to pick up biomarkers with potential health implications, we have focused on assessing the toxic and immunotoxic effects of the selected PETNPLs. Asa first conclusion, we have shown that MH-S cells efficiently internalize PETNPLs, with a 100% internalization at the concentration of 100 µg/mL. Our findings unequivocally demonstrate the harmful impact of PETNPLs on macrophage functions. This impact is evident through the disruption of the mitochondrial function, the elevated intracellular ROS levels, and the significant alterations in macrophage polarization, shifting the balance between M1 and M2 phenotypes. Due to the health relevance of the described biomarkers, our results underscore the need for further research, particularly focusing on factors such as NPL types, selected biomarkers, and cell types, to better understand the health implications associated with MNPL exposure. In addition to evaluating other MNPLs, other representative cell lung types such as the Calu-3 cells derived from human non-small-cell lung adenocarcinoma epithelial cells should be used. The advantage of this cell line is that can be used for the establishment of an *in vitro* lung epithelium barrier model since can display epithelial morphology expressing tight junctions and secreting abundant mucous substances. In addition, this cell line presents long- term stability which permits determining both acute and long-term ef- fects. In such a model, other relevant biomarkers should be included i.e., genotoxicity. It must be stated that genotoxicity has become an indis- pensable biomarker in any hazard evaluation. It is well known that DNA damage can drive very relevant health consequences such as gene/ chromosome mutation, carcinogenesis, and aging, among others but,

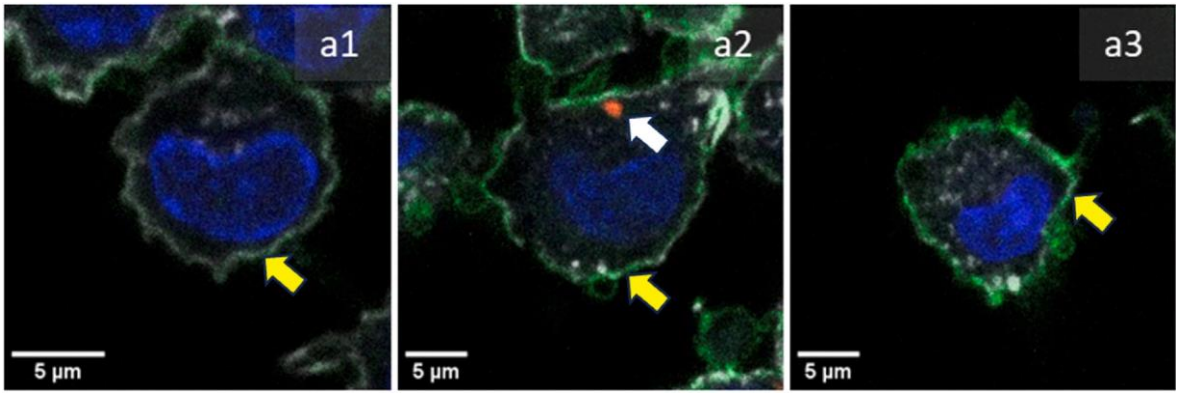


Fig. 6. MH-S cells were analyzed by confocal microscopy to detect CD86, as a surface marker of the M1 macrophage stage. (a1) Untreated control, (a2) cells with PETNPLs, and (a3) positive control cells exposed to LPS. The red spot indicates the internalized PETNPLs (white arrow), blue and gray colors are cell nuclei and actin, respectively. The green color indicates the expression of CD86 on the surface of the MH-S cells (a2 and a3), pointed out with yellow arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

ANNEXES

despite the relevance of this biomarker, very few studies have evaluatedthe potential genotoxic effects of MNPLs (Tagorti and Kaya, 2022).

CRediT authorship contribution statement

Alireza Tavakolpournegari: Investigation. **Aliro Villacorta:**Methodology. **Michelle Morataya-Reyes:** Methodology. **Jéssica Arri- bas Arranz:** Investigation. **Gooya Banaei:** Investigation. **Susana Pastor:** Investigation, Data curation. **Antonia Velázquez:** Validation, Investigation. **Ricard Marcos:** Writing – review & editing, Supervision, Conceptualization. **Alba Hernández:** Writing - review & editing, Funding acquisition, Conceptualization. **Balasubramanyam Annangi:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financialinterests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

AT and MMR hold Ph.D. fellowships from the Generalitat de Cata-lunya. AV was supported by a Ph.D. fellowship from the National Agency for Research and Development (ANID), CONICYT PFCHA/ DOCTORADO BECAS CHILE/2020 - 72210237. AH was granted an ICREA ACADEMIA award.
This project (Plasticheal) has received funding from the EuropeanUnion's Horizon 2020 research and innovation programme under grant agreement No 965196. This work was also partially supported by the Spanish Ministry of Science and Innovation [PID2020-116789, RB-C43] and by the Generalitat de Catalunya (2021-SGR-00731).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.123823.

References

Aguilar-Guzmán, J.C., Bejtka, K., Fontana, M., Valsami-Jones, E., Villezcas, A.M., Vazquez-Duhal, R., Rodríguez-Hernámdez, A.G., 2022. Polyethylene terephthalate nanoparticles effect on RAW 264.7 macrophage cells. Micropl. Nanopl. 2, 9. https://doi.org/10.1186/s43591-022-00027-1.
Allard, B., Panariti, A., Martin, J.G., 2018. Alveolar macrophages in the resolution of inflammation, tissue repair, and tolerance to infection. Front. Immunol. 9, 1777. https://doi.org/10.3389/fimmu.2018.01777.
Annangi, B., Villacorta, A., Vela, L., Tavakolpournegari, A., Marcos, R., Hernández, A., 2023. Effects of true-to-life PET nanoplastics using primary human nasal epithelialcells. Environ. Toxicol. Pharmacol. 100, 104140 https://doi.org/10.1016/j. etap.2023.104140.
Bao, X., Zhang, J., Huang, G., Yan, J., Xu, C., Dou, Z., Sun, C., Zhang, H., 2021. The crosstalk between HIFs and mitochondrial dysfunctions in cancer development. CellDeath Dis. 12 (2), 215. https://doi.org/10.1038/s41419-021-03505-1.
Bedard, K., Krause, K.H., 2007. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol. Rev. 87 (1), 245 - 313. https://doi.org/10.1152/physrev.00044.2005.
Bhutto, S.U.A., Ma, Y.F., Akram, M., You, X.Y., 2023. Microplastics in Tai lake food web: trophic transfer and human health risk assessment. Environ. Toxicol. Pharmacol. 101, 104206 https://doi.org/10.1016/j.etap.2023.104206.
Brandts, I., Solà, R., Garcia-Ordoñez, M., Gella, A., Quintana, A., Martin, B., Esteve-Codina, A., Teles, M., Roher, N., 2023. Polystyrene nanoplastics target lysosomes interfering with lipid metabolism through the PPAR system and affecting macrophage functionalization. Environ. Sci.: Nano 10, 2245 - 2258. https://doi.org/10.1039/D2EN01077A.
Carbery, M., O'Connor, W., Palanisami, T., 2018. Trophic transfer of microplastics and mixed contaminants in the marine food web and implications for human health. Environ. Int. 115, 400 - 409. https://doi.org/10.1016/j.envint.2018.03.007.
Chen, Q., Gao, J., Yu, H., Su, H., Yang, Y., Cao, Y., Zhang, Q., Ren, Y., Hollert, H., Si, H., Chen, C., Liu, H., 2022. An emerging role of microplastics in the etiology of lung ground glass nodules. Environ. Sci. Eur. 34 (1), 25. https://doi.org/10.1186/s12302-022-00605-3.
Ciaglia, E., Montella, F., Trucillo, P., Ciardulli, M.C., Di Pietro, P., Amodio, G., Remondelli, P., Vecchione, C., Reverchon, E., Maffulli, N., Puca, A.A., Della Porta, G., 2019. A bioavailability study on microbeads and nanoliposomes fabricated by dense carbon dioxide technologies using human-primary monocytes and flow cytometry assay. Int. J. Pharm. 570, 118686 https://doi.org/10.1016/j. ijpharm.2019.118686.
Collin-Faure, V., Dalzon, B., Devcic, J., Diemer, H., Cianférani, S., Rabilloud, T., 2022. Does size matter? A proteomics-informed comparison of the effects of polystyrene beads of different sizes on macrophages. Environ. Sci.: Nano 9, 2827 - 2840. https://doi.org/10.1039/d2en00214k.
Cortés, C., Domenech, J., Salazar, M., Pastor, S., Marcos, R., Hernández, H., 2020. Nanoplastics as potential environmental health factors. Effects of polystyrene nanoparticles on the human intestinal epithelial Caco-2 cells. Environ. Sci.: Nano 7, 272 - 285. https://doi.org/10.1039/c9en00523d.
Delaney, S., Rodriguez, C., Sarrett, S.M., Dayts, E.J., Zeglis, B.M., Keinänen, O., 2023. Unraveling the *in vivo* fate of inhaled micro- and nanoplastics with PET imaging. Sci.Total Environ. 904, 166320 https://doi.org/10.1016/j.scitotenv.2023.166320.
Deng, J., Ibrahim, M.S., Tan, L.Y., Yeo, X.Y., Lee, Y.A., Park, S.J., Wüstefeld, T., Park, J.W., Jung, S., Cho, N.J., 2022. Microplastics released from food containers can suppress lysosomal activity in mouse macrophages. J. Hazard Mater. 435, 128980 https://doi.org/10.1016/j.jhazmat.2022.128980.
Domenech, J., Hernández, A., Demir, E., Marcos, R., Cortés, C., 2020. Interactions of graphene oxide and graphene nanoplatelets with the *in vitro* Caco-2/HT29 model ofthe intestinal barrier. Sci. Rep. 10 (1), 2793. https://doi.org/10.1038/s41598-020-59755-0.
Fuchs, A.K., Syrovets, T., Haas, K.A., Loos, C., Musyanovych, A., Mailänder, V., Landfester, K., Simmet, T., 2016. Carboxyl- and amino-functionalized polystyrene nanoparticles differentially affect the polarization profile of M1 and M2 macrophage subsets. Biomaterials 85, 78 - 87. https://doi.org/10.1016/j. biomaterials.2016.01.064.
Gigault, J., El Hadri, H., Nguyen, B., Grassl, B., Rowenczyk, L., Tufenkji, N., Feng, S., Wiesner, M., 2021. Nanoplastics are neither microplastics nor engineered nanoparticles. Nat. Nanotechnol. 16 (5), 501 - 507. https://doi.org/10.1038/s41565-021-00886-4.
Harusato, A., Seo, W., Abo, H., Nakanishi, Y., Nishikawa, H., Itoh, Y., 2023. Impact ofparticulate microplastics generated from polyethylene terephthalate on gut pathology and immune microenvironments. iScience 26 (4), 106474. https://doi. org/10.1016/j.isci.2023.106474.
Jenner, L.C., Rotchell, J.M., Bennett, R.T., Cowen, M., Tentzeris, V., Sadofsky, L.R., 2022. Detection of microplastics in human lung tissue using μFTIR spectroscopy. Sci. Total Environ. 831, 154907 https://doi.org/10.1016/j.scitotenv.2022.154907.
Jomova, K., Raptova, R., Alomar, S.Y., Alwasel, S.H., Nepovimova, E., Kuca, K., Valko, M., 2023. Reactive oxygen species, toxicity, oxidative stress, and antioxidants: chronic diseases and aging. Arch. Toxicol. 97 (10), 2499 - 2574. https://doi.org/10.1007/s00204-023-03562-9.
Kalyanaraman, B., Darley-Usmar, V., Davies, K.J., Dennery, P.A., Forman, H.J., Grisham, M.B., Mann, G.E., Moore, K., Roberts, L.J., Ischiropoulos, H., 2012. Measuring reactive oxygen and nitrogen species with fluorescent probes: challengesand limitations. Free Rad. Biol. Med. 52 (1), 1 - 6. https://doi.org/10.1016/j. freeradbiomed.2011.09.030.
Karakolis, E.G., Nguyen, B., You, J.B., Rochman, C.M., Sinton, D., 2019. Fluorescent dyes for visualizing microplastic particles and fibers in laboratory-based studies. Environ.Sci. Technol. Lett. 6 (6), 334 - 340. https://doi.org/10.1021/acs.estlett.9b00241.
Kim, L., Cui, R., Il Kwak, J., An, Y.J., 2022. Trophic transfer of nanoplastics through a microalgae-crustacean-small yellow croaker food chain: inhibition of digestive enzyme activity in fish. J. Hazard Mater. 440, 129715 https://doi.org/10.1016/j.jhazmat.2022.129715.
Koner, K., Florance, I., Mukherjee, A., Chandrasekaran, N., 2023. Cellular response of THP-1 macrophages to polystyrene microplastics exposure. Toxicology 483, 153385. https://doi.org/10.1016/j.tox.2022.153385.
Kumar, R., Manna, C., Padha, S., Verma, A., Sharma, P., Dhar, A., Ghosh, A., Bhattacharya, P., 2022. Micro(nano)plastics pollution and human health: How plastics can induce carcinogenesis to humans? Chemosphere 298, 134267. https://doi.org/10.1016/j.chemosphere.2022.134267.
Latroche, C., Weiss-Gayet, M., Gitiaux, C., Chazaud, B., 2018. Cell sorting of various cell types from mouse and human skeletal muscle. Methods 134 - 135, 50 - 55. https://doi.org/10.1016/j.ymeth.2017.12.013.
Le, V.G., Nguyen, M.K., Nguyen, H.L., Lin, C., Hadi, M., Hung, N.T.Q., Hoang, H.G., Nguyen, K.N., Tran, H.T., Hou, D., Zhang, T., Bolan, N.S., 2023. A comprehensive review of micro- and nano-plastics in the atmosphere: occurrence, fate, toxicity, andstrategies for risk reduction. Sci. Total Environ. 904, 166649 https://doi.org/ 10.1016/j.scitotenv.2023.166649.
Lim, D., Jeong, J., Song, K.S., Sung, J.H., Oh, S.M., Choi, J., 2021. Inhalation toxicity of polystyrene micro(nano)plastics using modified OECD TG 412. Chemosphere 262, 128330. https://doi.org/10.1016/j.chemosphere.2020.128330.
Lin, S., Zhang, H., Wang, C., Su, X.L., Song, Y., Wu, P., Yang, Z., Wong, M.H., Cai, Z., Zheng, C., 2022. Metabolomics reveal nanoplastic-induced mitochondrial damage inhuman liver and lung cells. Environ. Sci. Technol. 56 (17), 12483 - 12493. https://doi.org/10.1021/acs.est.2c03980.

ANNEXES

Murray, P.J., 2017. Macrophage polarization. Annu. Rev. Physiol. 79, 541-566. https://doi.org/10.1146/annurev-physiol-022516-034339.

Nanogenotox project, 2011. Final protocol for producing suitable manufactured nanomaterial exposure media. Nanogenotox, towards a method for detecting the potential genotoxicity of nanomaterials. https://www.anses.fr/en/system/files/nanogenotox_deliverable_5.pdf. (Accessed 15 June 2023).

Nguyen, B., Tufenkji, N., 2022. Single-particle resolution fluorescence microscopy of nanoplastics. Environ. Sci. Technol. 56 (10), 6426-6435. https://doi.org/10.1021/acs.est.1c08480.

Pérez, S., Rius-Pérez, S., 2022. Macrophage polarization and reprogramming in acute inflammation: a redox perspective. Antioxidants 11 (7), 1394. https://doi.org/10.3390/antiox11071394.

Pulvirenti, E., Ferrante, M., Barbera, N., Favara, C., Aquilia, E., Palella, M., Cristaldi, A., Conti, G.O., Fiore, M., 2022. Effects of nano and microplastics on the inflammatoryprocess: *in vitro* and *in vivo* studies systematic review. Front. Biosci. 27 (10), 287. https://doi.org/10.31083/j.fbl2710287.

Rubio, L., Marcos, R., Hernández, A., 2020. Potential adverse health effects of ingested micro- and nanoplastics on humans. Lessons learned from *in vivo* and *in vitro* mammalian models. **J.** Toxicol. Environ. Health B Crit. Rev. 23 (2), 51-68. https://doi.org/10.1080/10937404.2019.1700598.

Sakamuru, S., Attene-Ramos, M.S., Xia, M., 2016. Mitochondrial membrane potential assay. Methods Mol. Biol. 1473, 17-22. https://doi.org/10.1007/978-1-4939-6346- 1_2.

Senathirajah, K., Attwood, S., Bhagwat, G., Carbery, M., Wilson, S., Palanisami, T., 2021. Estimation of the mass of microplastics ingested - a pivotal first step towards humanhealth risk assessment. J. Hazard Mater. 404 (Pt B), 124004 https://doi.org/10.1016/j.jhazmat.2020.124004.

Stock, V., Laurisch, C., Franke, **J.,** Dönmez, M.H., Voss, L., Böhmert, L., Braeuning, A., Sieg, H., 2021. Uptake and cellular effects of PE, PP, PET, and PVC microplastic particles. Toxicol. Vitro 70, 105021. https://doi.org/10.1016/j.tiv.2020.105021.

Suski, J., Lebiedzinska, M., Bonora, M., Pinton, P., Duszynski, J., Wieckowski, M.R., 2018. Relation between mitochondrial membrane potential and ROS formation. Methods Mol. Biol. 1782, 357-381. https://doi.org/10.1007/978-1-4939-7831-1_22.

Tagorti, G., Kaya, B., 2022. Genotoxic effect of microplastics and COVID-19: the hidden threat. Chemosphere 286 (Pt 3), 131898. https://doi.org/10.1016/j.chemosphere.2021.131898.

Tavakolpournegari, A., Annangi, B., Villacorta, A., Banaei, G., Martin, J., Pastor, S.,Marcos, R., Hernández, A., 2023. Hazard assessment of different-sized polystyrene nanoplastics in three hematopoietic human cell lines. Chemosphere 325, 138360. https://doi.org/10.1016/j.chemosphere.2023.138360.

Tursi, A., Baratta, M., Easton, T., Chatzisymeon, E., Chidichimo, F., De Biase, M., De, Filpo G., 2022. Microplastics in aquatic systems, a comprehensive review: origination, accumulation, impact, and removal technologies. RSC Adv. 12 (44), 28318-28340. https://doi.org/10.1039/d2ra04713f.

Van den Bossche, J., Baardman, J., Otto, N.A., van der Velden, S., Neele, A.E., van den Berg, S.M., Luque-Martin, R., Chen, H.J., Boshuizen, M.C., Ahmed, M., Hoeksema, M.A., de Vos, A.F., de Winther, M.P., 2016. Mitochondrial dysfunction prevents repolarization of inflammatory macrophages. Cell Rep. 17 (3), 684-696. https://doi.org/10.1016/j.celrep.2016.09.008.

Villacorta, A., Rubio, L., Alaraby, M., López-Mesas, M., Fuentes-Cebrian, V., Moriones, O. H., Marcos, R., Hernández, A., 2022. A new source of representative secondary PET nanoplastics. Obtention, characterization, and hazard evaluation. J. Hazard Mater. 439, 129593 https://doi.org/10.1016/j.jhazmat.2022.129593.

Villacorta, A., Vela, L., Morataya-Reyes, M., Llorens-Chiralt, R., Rubio, L., Alaraby, M.,Marcos, R., Hernández, A., 2023. Titanium-doped PET nanoplastics of environmental origin as a true-to-life model of nanoplastic. Sci. Total Environ. 880, 163151 https://doi.org/10.1016/j.scitotenv.163151.

Wang, Y., Li, N., Zhang, X., Horng, T., 2021. Mitochondrial metabolism regulates macrophage biology. J. Biol. Chem. 297 (1), 100904 https://doi.org/10.1016/j.jbc.2021.100904.

Wang, X., Ren, X.M., He, H., Li, F., Liu, K., Zhao, F., Hu, H., Zhang, P., Huang, B., Pan, X., 2023. Cytotoxicity and pro-inflammatory effect of polystyrene nano-plastic and micro-plastic on RAW264.7 cells. Toxicology 484, 153391. https://doi.org/10.1016/j.tox.2022.153391.

Washihira, N., Murakami, M., Nakamura, M., Fujii, S., Matsushima, T., Asahara, H., Kishida, A., Tanabe, T., Kimura, T., Kobayashi, M., Yamamoto, M., 2023. Applicationof a genetically engineered macrophage cell line for evaluating cellular effects of UV/US-treated poly (ethylene terephthalate) microplastics. Colloids Surf. B Biointerfaces 234, 113735. https://doi.org/10.1016/j.colsurfb.2023.113735.

Wolff, C.M., Singer, D., Schmidt, A., Bekeschus, S., 2023. Immune and inflammatory responses of human macrophages, dendritic cells, and T-cells in presence of micro-and nanoplastic of different types and sizes. J. Hazard Mater. 459, 132194 https://doi.org/10.1016/j.jhazmat.2023.132194.

Wu, Y., Yao, Y., Bai, H., Shimizu, K., Li, R., Zhang, C., 2023. Investigation of pulmonary toxicity evaluation on mice exposed to polystyrene nanoplastics: the potential protective role of the antioxidant N-acetylcysteine. Sci. Total Environ. 855, 158851.

Xu, L., Yan, X., Zhao, Y., Wang, J., Liu, B., Yu, S., Fu, J., Liu, Y., Su, J., 2022. Macrophage polarization mediated by mitochondrial dysfunction induces adipose tissue inflammation in obesity. Int. J. Mol. Sci. 23 (16), 9252.

Yang, S., Cheng, Y., Chen, Z., Liu, T., Yin, L., Pu, Y., Liang, G., 2021. In vitro evaluation of nanoplastics using human lung epithelial cells, microarray analysis and co-culture model. Ecotoxicol. Environ. Saf. 226, 112837 https://doi.org/10.1016/j. ecoenv.2021.112837.

Zhang, H., Zhang, S., Duan, Z., Wang, L., 2022. Pulmonary toxicology assessment of polyethylene terephthalate nanoplastic particles *in vitro*. Environ. Int. 162, 107177 https://doi.org/10.1016/j.envint.2022.107177.

Zingaro, F., Gianoncelli, A., Ceccone, G., Birarda, G., Cassano, D., La Spina, R., Agostinis, C., Bonanni, V., Ricci, G., Pascolo, L., 2023. Morphological and lipid metabolism alterations in macrophages exposed to model environmental nanoplastics traced by high-resolution synchrotron techniques. Front. Immunol. 14, 1247747 https://doi.org/10.3389/fimmu.2023.1247747.

ANNEXES

Update

Environmental Pollution

Volume 349, Issue , 15 May 2024, Page

DOI:
https://doi.org/10.1016/j.envpol.2024.123996

ANNEXES

ANNEXES

7.2.1. The pdf of the corrigendum to “Harmful effects of true-to-life nanoplastics derived fromPET water bottles in human alveolar macrophages”

Environmental Pollution 349 (2024) 123996

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage:

Corrigendum to “Harmful effects of true-to-life nanoplastics derived fromPET water bottles in human alveolar macrophages” [Environ. Pollut., **348**: 123823 (2024)]

A. Tavakolpournegari, A. Villacorta, M. Morataya-Reyes, J. Arribas Arranz, G. Banaei, S. Pastor, A. Velázquez, R. Marcos *, A. Hernández, B. Annangi

Universitat Autònoma de Barcelona, Spain

The authors regret the presence of the word ‘human’ in the title. The correct word is ‘mouse’.

The authors would like to apologise for any inconvenience caused

Available online 18 April 2024

DOI of original article: https://doi.org/10.1016/j.envpol.2024.123823.

* Corresponding author.

E-mail address: ricard.marcos@uab.es (R.Marcos).

https://doi.org/10.1016/j.envpol.2024.123996

ANNEXES

7.2.2. Supplementary figures related to the published paper entitled Harmful effects of true-to-life nanoplastics derived from PET water bottles in alveolar macrophages

Harmful effects of true-to-life nanoplastics derived from PET water bottles in human alveolar macrophages

Alireza Tavakolpournegari[1], Aliro Villacorta[1,2], Michelle Morataya-Reyes[1], Jéssica Arribas Arranz[1], Gooya Banaei[1], Susana Pastor[1], Antonia Velázquez[1], **Ricard Marcos[1], Alba Hernández[1,*] Balasubramanyam Annangi[1,*],**

SUPPLEMENTARY MATERIAL

ANNEXES

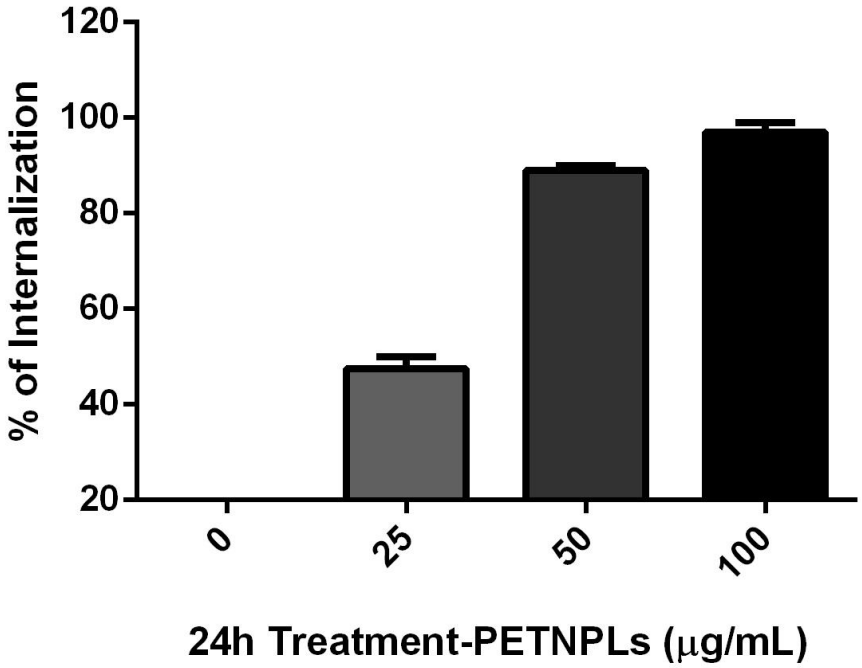


Figure S1. The percentage of positive sorted cells from the live population exposed to three different concentrations of iDye-labeled PETNPLs.

ANNEXES

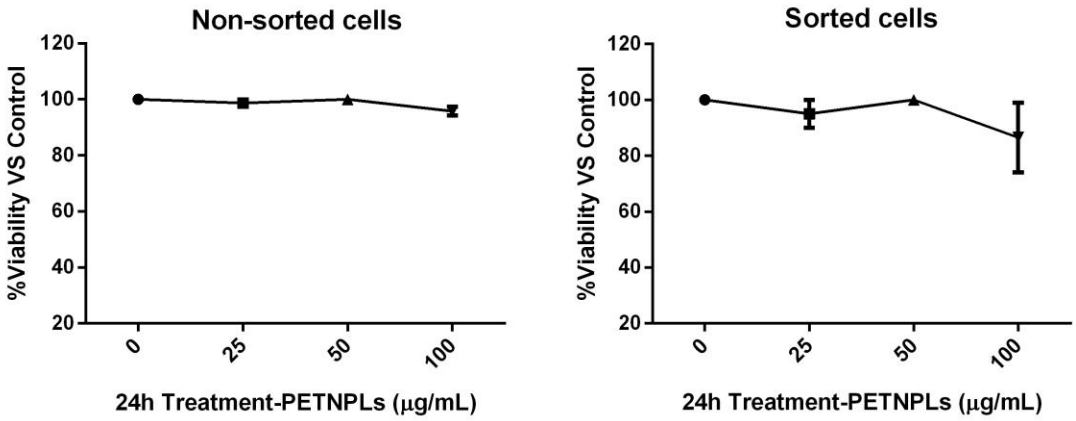


Figure S2. The graph shows the viability of MH-S cells containing PETNPLs after exposures lasting for 24 h for both sorted and non-sorted cells.